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(54) Title: PHARMACEUTICAL PREPARATIONS OF BIOACTIVE SUBSTANCES EXTRACTED FROM NATURAL SOURCES

(57) Abstract: This invention relates to methods of extracting and purifying bioactive substances from various plants and herbs. More specifically the invention relates to methods of extracting and separating bioactive substances from various plants and herbs, such as Kava root, Byrsonima species, Aesculus californica, Crataegus mexicana, Simmondsia chinensis, Pfaffia species, Alternanthera repens, Bursera species, Turnera species, Perezia species, Heimia salicifolia, Psidium species, Enterlobium species, Ptychopetalum olacoides, Liriosma ovata, and Chaunochiton kappleri, using supercritical fluid extraction and/or fluorocarbon solvent extract. The present invention further relates to separation of bioactive substances contained in extracts using packed column supercritical fluid chromatography or HPLC where dense gas with or without modifiers is the mobile phase. The present invention also relates to pharmaceutical preparations and dietary supplements which may be prepared with the extracted bioactive substances and use of such pharmaceutical preparations and dietary supplements to treat various human ailments.

PHARMACEUTICAL PREPARATIONS OF BIOACTIVE SUBSTANCES EXTRACTED FROM NATURAL SOURCES

Background

5 1. Field of the Invention

This invention relates to methods of extracting and purifying bioactive substances from various plants and herbs. More specifically the invention relates to methods of extracting and separating bioactive substances from various plants and herbs using supercritical fluid extraction and/or fluorocarbon solvent extract. The present
10 invention further relates to separation of bioactive substances contained in extracts using packed column supercritical fluid chromatography. The present invention also relates to formulations, pharmaceutical preparations and dietary supplements which may be prepared with the extracted bioactive substances and use of such pharmaceutical preparations and dietary supplements to treat various human ailments.

15 2. Description of the Background

Throughout history humans have ingested and otherwise consumed a wide variety of plants and herbs, and extracts of such plants and herbs to help alleviate aches and pains, improve immunity to infection, treat various illnesses, or even to induce relaxation or stress reduction.

20 One plant that has been commonly ingested by the people of the South Pacific to induce relaxation is called Kava Root. K. Schubel, J. Soc. Chem. Ind., 43, 766 (1924); A. G. Van Veen, Rec. Trav. Chim., 58, 52 (1939). Kava root consists of the dried rootstock and/or shoots of *Piper methysticum* Forst (Family: Piperaceae). The Kava root is most typically ingested by drinking an aqueous macerate (pulverized Kava
25 root mixed with water) known as the beverage Kava.

First attempts to identify the active compounds within Kava root were made over a hundred years ago. Those efforts resulted in the identification of kavalactones, also known as kavapyrones. More than ten kavalactones as well as four other substances have been identified in the Kava root to date, including kavain, dihydrokavain
30 (a.k.a. marindinin), methysticin, dihydromethysticin, yangonin, and desmethoxyyangonin. V. Lebot, M. Merling, and L. Lindstrom, "Kava the Pacific Drug", Yale University Press, New Haven, CT (1992). These compounds are neutral, nitrogen-poor

compounds that may be specifically referred to as substituted d-lactones and substituted a-pyrones. The lactone ring is substituted by a methoxy group in the C3 position, and the differences in the compounds lie in the degree of unsaturation (e.g. yangonin, desmethoxyyangonin, kavain and methysticin) or by benzene substitution (e.g. dihydrokavain and dihydromethysticin), as shown in Figure 24.

The particular kavalactones in a Kava root extract vary depending upon its origin. Different species of kavalactones have been found to have varying physiological effects in vivo depending on their molecular structure. All naturally occurring kavalactones contain an enolic double bond between C3 and C4. The dienolides of the yangonin type appear to be pharmacologically inert. In the enolides, the effective optimum varies as a function of the hydrogenation of the double-bonded C7. For example, kavain has the strongest effect as a local anesthetic, dihydromethysticin as a spasmolytic, and dihydrokavain as an intensifier of narcosis. R. Hansel, Characterization and Physiological Activity of Some Kava Constituents Pacific Science, July 1968, Vol. XXII: pp293-313.

Further, the particular kavalactones present depend upon whether, in addition to rhizome parts, roots and stems of the plant are included in the extract. High quality extracts of the Kava root are sold based upon the total kavalactone content, rather than upon analysis of the individual lactones contained therein. The concentration ranges of total kavalactone levels in the Kava root extracts employed, e.g. in Germany are generally within the range of 30 to 55 weight percent.

Although many types of kavalactones have been identified, no simple and efficient method is available for both extraction of the root and separation of each individually extracted lactone. The traditional extraction method (e.g. steam distillation) usually involved mixing 100 grams of root with a suitable quantity of distilled water producing a slurry having a volume of approximately 200 mL. A. R. Furguele, W. J. Kinnard, M.D. Aceto, and J. P. Buckley, J. Pharmaceutical Sci., 54, 248 (1965). The slurry was steam distilled and the first 100 mL of distillate was collected, filtered and lyophilized. The yield for each extraction was about 50 mg. Alternately, a liquid-solid extraction at room temperature has been reported wherein the above slurry was

intimately mixed in a Waring blender for 15 minutes. The mixture was then filtered and lyophilized. In certain cases, rather than lyophilization, the filtrate was subjected to successive extractions with chloroform. This purification operation basically removed impurities from the aqueous layer. The extraction yield for these methods varied
5 depending on the solvent and methodology used.

Modern Kava root extracts are commonly manufactured using ethanol as a solvent because kavalactones are readily soluble in ethanol. The extractable materials are in the form of a yellowish brown paste or powder, which is then tested to assure proper concentrations of kavalactones.

10 A plant that has been commonly ingested by the people of Mexico and other Latin American countries is *Byrsonima crassifolia* (Nanche). The medicinal importance of this tropical tree, which is indigenous to Mexico, has been documented historically since the sixteenth century. Traditional healers use the plant to treat gastrointestinal disorders, especially diarrhea and dysentery.

15 To date, about 21 chemical substances have been extracted from the dried leaves and bark of the tree, including β -sitosterol and betulin (triterpenes), pipecolic acid and proline (amino acids), and catechin and quercetin (flavonoids). Béjar, E., et al., Constituents of *Byrsonima crassifolia* and their spasmogenic activity, *Int. J. Pharmacog.* 1995, 33:1, 25-32. The discovery of pipecolic acid is significant in that it is a rare
20 compound in nature and is an important intermediate in a number of pharmacological preparations which demonstrate therapeutic effect for stroke, Parkinson's disease, Alzheimer's disease, and other neurological and vascular disorders. Prior to the discovery of pipecolic acid in *Byrsonima crassifolia*, preparations containing pipecolic acid were derived from various cultured micro-organisms.

25 Traditional healers prepared aqueous solutions of *Byrsonima* as teas. It was recently discovered that aqueous extracts of *Byrsonima* contain only catechin. However, when methanol is used to extract bioactive substances from *Byrsonima*, a wide variety of triterpenes, amino acids and flavonoids can be isolated.

Plants in the genera *Aesculus* and *Crataegus* are known to contain bioactive
30 substances which affect the heart and circulatory system. Galenical preparations of, for

example, *Crataegus oxyacantha*, *C. azarolus*, *C. monogyna*, *C. pentagyna*, *C. laevigata* and *C. nigra* have been used in European herbalism for centuries for these purposes. *Crataegus pinnatifida* has been used for similar purposes in Traditional Chinese Medicine for even longer. Likewise the use of *Aesculus hippocastanum* in Europe for the treatment of circulatory disorders is well documented. The effect has been attributed to aescin, a mixture of triterpene glycosides which have an anti-exudative and vascular tightening effect. While these European and Asian species have been the subject of a great deal of research, co-generic species endemic to the New World have been largely ignored. *Aesculus californica*, commonly known as 'California buckeye' in English and 'berruco' in Spanish, had been used by the native tribes and early colonists of California for a variety of purposes. The dried bark of the tree was used for toothaches, the fresh seeds were eaten after leaching out the bitter principles, and the unprocessed fruits were used to treat hemorrhoids, as a fish poison, and as an abortifacient.

Analyses of the seeds of *Aesculus californica* by several groups have revealed the presence of a number of known bioactive compounds: the proteids β -methyl alanine, phenylalanine, isohomoleucine, isohomo-6-hydroxyleucine, mino-4-methyl-hex-trans-4-enoic acid and gamma-glutamyl-2-A-hex-4-enoic acid; the benzoids arbutin and hydroquinone; the flavonoid epicatechin; and the coumarin eleutheroside B-1, as well as the carbohydrate quebrachitol. This chemical profile differs from the European *A. hippocastanum*.

Extracts of *Crataegus* and *Aesculus* species are commonly prepared using various solvents, such as methanol, ethanol or acetone. The extracts are taken from the leaves and flowers of *Crataegus* species and from the seeds, leaves and bark of the *Aesculus* species.

The plant *Simmondsia chinensis*, also known as Jojoba, is native to the desert areas of the Southwestern United States and Mexico. Jojoba has a unique wax ester oil which is 50 to 60% of its seed weight. This oil is currently used in cosmetics and lubricants. The remainder of the seed is not used as much as the oil although it contains about 25% crude protein after the oil is removed. The defatted meal contains sugars and 11 to 15% of a unique group of natural products.

Simmondsin, one of the natural products contained in Jojoba meal, has been shown to be an effective hunger satiation agent by reducing food intake in mice, rats, and chickens. Cokeleare et al. (1995, Ind. Crops Prod., 4:91-96). Simmondsin has also been shown to be a useful weight reduction agent for Dogs. See U.S. Pat. No. 5,962,043. However, Jojoba meal also contains other antinutritional factors such as trypsin inhibitor, polyphenols, bitter taste, nonnutritive protein, and indigestible Jojoba oil.

Methods of removing so-called "toxic" principles from Jojoba seed meal in order to render it palatable to animals as feed have been described. See U.S. Pat. No. 5,672,371 to d'Oosterlynck, U.S. Pat. No. 4,209,534 to Banigan et al., and U.S. Pat. No. 4,148,928 to Sodini. Also, solvents have been used to extract simmondsins from Jojoba meal. U.S. Pat. No. 6,007,823.

Pfaffia paniculata, commonly called Brazilian ginseng, is a plant in the family *Amaranthaceae* which grows in parts of Brasil, Paraguay, Uruguay and Argentina. All parts of the plant are used in folk medicine, but it is the roots that are considered most valuable medicinally. Traditionally, the plant has been used to treat diabetes, rheumatism, ulcers, leukemia and other cancers, and as a tranquilizer, general tonic, and aphrodisiac.

Recent studies have demonstrated that the plant has biological activity as an anti-allergenic, analgesic, anti-inflammatory, antitumor agent, has a weak CNS-depressant effect and decreases vascular permeability. The plant has further been shown to be non toxic to humans.

Extracts of the plant have been shown to contain allantoin, daucosterol, b-ecdysone, pfaffic acid, pfaffosides A, B, C, D, E, and F, polypodine B, β -sitosterol, stigmasterol, and stigmasterol-3-O- β -D-glucoside.

Turnera diffusa and other *Turnera* species, commonly called damiana, hierba del venado, and other names, are small, herbaceous perennials ranging from California to South America. The plant has been used since pre-Columbian times as an aphrodisiac and sexual tonic, expectorant, diuretic, antidiabetic, to increase fertility, treat spermatorrhea, orchitis, nephritis, chronic coughing, and as a stimulant, digestive aid,

and laxative. Laboratory tests of various *Turnera* preparations have shown cytotoxic and antihyperglycemic effects. The plant extract has been found to be non-mutagenic.

Turnera species are known to contain arbutin, caffeine, gonzalitosin, β -sitosterol, an acetovanillin-like benzenoid compound, hexacosan-1-ol, tetraphyllin B, N-triacontane, tricosan-2-one, an essential oil which contains 1-8-cineol, paracymene, a-pinene, b-pinene, and three sesquiterpenes.

The roots of plants of the genus *Perezia* produce perezone (2-(1,5-dimethyl-4-hexenyl)-3-hydroxymethyl-p-benzoquinone). Perezone is a sesquiterpenic benzoquinone which exhibits oxido-reduction characteristics. Certain species of the *perezia* genus have been used as laxatives in Mexican folk medicine.

In studies of the effect of perezone on electron transport in biological membranes, it was found that perezone inhibits mitochondrial electron transport in rat liver mitochondria differently than rotenone, amytal, and Antimycin A. Carabez A. et al., The Action of the Sesquiterpenic Benzoquinone, Perezone, on Electron Transport in Biological Membranes. Arch Biochem Biophys. 1988 Jan; 260(1):293-300. The low respiration of rat liver mitochondria depleted of coenzyme Q10 (CoQ) was shown to be increased by perezone.

Heimia salicifolia was used as a traditional medicine in the Americas to treat inflammation. In recent studies, two alkaloids from *Heimia salicifolia*, cryogenine and nesodine, were discovered to be more than twice as potent as aspirin as inhibitors of prostoglandin synthetase prepared from bovine seminal vesicles.

In-vitro-grown shoots of *Heimia salicifolia* have been found to be active in alkaloid biosynthesis, yielding the biphenylquinolizidine lactones vertine, lytrine, and lyfoline, the ester alkaloids demethoxyabresoline and epidemethoxylabresoline, the phenylquinolizidinols demethyllasubine-I and demethyllasubine-II. Rother, A., The phenyl- and biphenyl-quinolizidines of in-vitro-grown *Heimia salicifolia*. J. Nat. Prod. 1985 Jan-Feb; 48(1):33-41. Five to ten day old seedlings of *Heimia salicifolia* have also been used to extract bioactive species. Two isomeric 2-hydroxy-4-(3-hydroxy-4-methoxyphenyl) quinolizidines, differing in the configuration of the bridgehead carbon, have been isolated by Rother, A. et al. Radioactive dilution

has been used to isolate 2-keto-4- (3-hydroxy-4-methoxyphenyl)quinolizidine from the seedlings.

Although these and many other plant species are known for various therapeutic and healing effects, these plants have further benefits, and synergistic effects when multiple plants are combined, that have not yet been described. The bioactive substances which make these plants medicinally effective are commonly extracted with solvents and/or water. This technology has several disadvantages among which are the cost of the solvents, costs associated with their safe disposal, and removal of the solvents from the extract.

Furthermore, medicinal plant chemistry is complex and the vast majority of medicinal plants owe their pharmacological action to many different molecular entities which often belong to more than one class of compounds. Many solvents have only a limited effectiveness for eluting certain classes of compounds, resulting in inefficient extractions. These types of extractions generally result in low concentrations of bioactive substances and a need for multiple extractions with different solvents to isolate differing substances.

An extraction method which removes high concentrations of multiple bioactive substances is desirable. A separation method which permits efficient separation of the substances to obtain purified, therapeutically effective quantities of bioactive substances is also desired. Such methods would provide new extracts from known plant species, the ability to isolate useful quantities of specific bioactive substances, new uses of extracts from known plant species, and more efficient extraction.

Supercritical fluid extraction and supercritical fluid chromatography have been used in the chemical arts for many years. Gases such as carbon dioxide or propane have proven to have excellent solvating properties when pressurized, particularly above their critical point. This so-called supercritical region occurs when a gas is pressurized to a point where it would normally liquify, but is simultaneously heated above its now greatly reduced boiling point to prevent liquification. This "supercritical fluid" is neither a liquid nor a gas, but exhibits properties of both. In particular, supercritical fluids

possess excellent solvating properties with high selectivity for particular analytes. This selectivity can be further adjusted by variations of pressure, temperature and use of mixed gases.

Lopez and Benedicto used supercritical CO₂ to extract kavalactones from Kava herb. V. Lopez-Avila and J. Benedicto, J. High Resolut. Chromatogr., 20, 555 (1997). In each extraction a 10 mL cartridge was filled with 2.5 grams of Kava herb which was extracted with both pure and 15% ethanol-modified CO₂ for a dynamic extraction time of 60 minutes at 250 atm and 60°C. Extracted analytes were collected in a vial filled initially with 4 mL of ethanol maintained at 22°C. Recovery was less than 25% when pure CO₂ was used as the extraction fluid, but was greater than 90% (relative to a solid-liquid extraction) when using 15% ethanol-modified CO₂. Identification of each of the extracted kavalactones was determined via GC/MS. Not only was the supercritical fluid extraction highly efficient, but there were very few co-extractives.

Although CO₂ proved generally effective for extraction of kavalactones, CO₂ only works as an extraction medium at extreme pressures, generally on the order of several thousands of pounds per square inch. This factor contributes to the high cost of equipment and to inherent dangers associated with extreme pressure vessels. Various types of chromatography have been used to separate and determine the major constituents of Kava extracts. Nakayama et al. used thin layer chromatography to separate and quantify six kavalactones (R. L. Young, J. W. Hylin, D. L. Plucknett, Y. Kawano, and R. T. Nakayama. *Phytochemistry*, 5, 795 (1966)). Later, Gracza et al. used normal phase high pressure liquid chromatography (HPLC) to separate a mixture of kavalactones (L. Gracza and P. Ruff, *J. Chromatogr.*, 486, 193 (1980)). Haberlein et al. have also used normal phase HPLC to separate and quantify a series of kavalactones (H. Haberlein, G. Boonen, and M. A. Beck, *Planta Med.* 63, 63 (1997); G. Boonen, M. A. Beck, and H. Haberlein, *J. Chromatogr. B*, 702, 240 (1997)). Reverse phase HPLC was used to separate kavalactones, however, most of the separations were poor. R. M. Smith, H. Thakrar, T. A. Arowolo, and A. A. Shafi J. *Chromatogr.*, 283, 303 (1984). Recently, Shao et al. used reverse phase HPLC with atmospheric pressure chemical ionization mass spectrometry in the positive ion mode to

separate and identify specific kavalactones. Baseline separation of six lactones was achieved in less than 36 minutes. Y. Shao, K. He, B. Zheng, and Q. Zheng. J. Chromatogr. A, 825, 1 (1998).

Although some of these methods have proven fairly efficient for identifying, obtaining, separating, and isolating various kavalactones, improvements to the field are necessary. Additionally, a method for simply and accurately obtaining, separating and isolating different species of bioactive substances from other plant species are still lacking. Furthermore, in today's health conscious society, novel applications of natural source substances, and methods for obtaining such therapeutically useful substances, are necessary. **Summary of the Invention**

The present invention overcomes the problems and disadvantages associated with current strategies and designs and provides novel methods for extracting, separating, and isolating bioactive substances from natural sources. The present invention further relates to novel therapeutic uses of such extracts.

Accordingly, one embodiment of the invention is directed to methods for the preparative and/or commercial scale extraction of bioactive substances comprising the step of using supercritical fluid extraction (SFE) or near-critical extraction (NCE) for said preparative and/or commercial-scale extraction. The SFE or NCE may be accomplished with CO₂ or CO₂ modified with various other volatile substances. The SFE or NCE may further be accomplished as a batch-wise extraction, continuous-cascading extraction, or countercurrent-solvent extraction.

Another embodiment is directed to methods for the preparative and/or commercial scale processing of bioactive substances comprising coupling SFE or NCE and supercritical fluid chromatography (SFC), with or without modifiers, for said preparative and/or commercial scale processing. In this embodiment, isopropyl amine may be used as a modifier in SFC.

Another embodiment of the invention is directed to methods for the preparative and/or commercial scale extraction of bioactive substances comprising the step of using dense gases in the supercritical, near critical, or subcritical state with or without modifiers, for said preparative and/or commercial scale extraction. The dense

gas may be any non-chlorinated fluorocarbon solvent and the modifiers may be any other volatile substance. The extraction may be performed under a pressure of 0-10 bar, or under supercritical or near critical fluid conditions. Dense gas extraction may further be accomplished as a batch-wise extraction, continuous-cascading extraction or
5 countercurrent-solvent extraction

Another embodiment of the invention is directed to methods for the separation of bioactive substances comprising the step of SFC. The step of using SFC preferably comprises the use of HH₂ and/or C4 columns, singly or in combination, in the SFC separation.

10 Another embodiment of the invention is directed to compositions comprising medicinal formulations of extracts of *Byrsonima* species recovered with supercritical fluid extraction and/or dense gases or with various organic solvents and/or water, and to methods of administering therapeutically effective amounts of these formulations to patients in need of treatment. *Byrsonima* species extracts are used alone or are combined
15 with advantageous effect with various *Psidium* and *Enterolobium* species extracts, which are similarly prepared. Compositions may comprise extracts or isolated products of *Aesculus californica* and *Crataegus mexicana*, either on their own, in combination with one another, or in combination with extracts from various *Bursera* species.

Another embodiment of the invention is directed to extraction of simmondsin
20 compounds from *Jojoba* (*Simmondsia chinensis*) and use of these compounds as a human weight loss agent.

Another embodiment of the invention is directed to formula and compositions comprising a combination of extracted phytochemicals from *Turnera* species and *Pfaffia* species, with or without *muira puama* (a crude drug derived from various species
25 including *Ptychopetalum olacoides*, *Liriosma ovata*, and *Chaunochiton kappleri*) for use as a health tonic and to support sexual function.

Another embodiment of the invention is directed to formula and compositions comprising a combination of extracted phytochemicals from, for example, *Heimia salicifolia*, for use as a Non-steroidal Anti-inflammatory Drug (NSAID).

Other embodiments and advantages of the invention are set forth in part in the description which follows, and in part, will be obvious from this description, or may be learned from the practice of the invention.

Description of the Drawings

5 Figure 1 Gas Chromatograph/Mass Spectroscopy (GC/MS) separation of kavalactones extracted using SFE.

Figures 2-8 Mass spectra of each kavalactone listed in Table 2.

Figures 9-13 Spectra of other major peaks which eluted before kavalactones (tR = 19.40, 22.1, 23.01, 24.35, and 26.93 min).

10 Figure 14 GC/MS chromatogram of kavalactones extracted via sonication.

Figures 15-17 Results of experiments wherein kavalactone extracts were subjects to SFC using NH₂, DIOL, and CN columns.

Figure 18 Results of an experiment wherein kavalactone SFE extracts were separated with SFC at a higher temperature (80°C) using the CN column. All other
15 chromatography conditions were the same as described for CN above.

Figure 19 Pressure (125 atm) did not change the selectivity of the column.

Figures 20 and 21 Separation of kavalactone extracts on NH₂ columns at 40°C and 80°C, respectively.

Figure 22 Results of a separation of the same Kava root extract on an NH₂ column,
20 using the same conditions as described with Figure 21, with the exception that pressure was increased to 275 atm.

Figure 23 For this experiment the same pressure (125 atm), temperature (80°C), flow (2mL/min of liquid CO₂), and column (NH₂) was used with the exception that the modifier programming started with 7/93% MeOH/CO₂ hold for 3 minutes
25 and then increased to 10/90% CO₂/MeOH at the rate of 0.2% minutes.

Figure 24 Chemical structure of seven identified kavalactones.

Figure 25 SFC separation of kavalactone extract. Column NH₂ (250 x 4.6 mm. 5µm dp). Pressure 125 atm, 60°C, 2 mL/min. Modifier program: 98/2% CO₂/MeOH for 3 min. and then increased to 90/10 CO₂/MeOH at rate of
30 9.4%/min.

Figure 26 SFC separation of kavalactone extract. Column NH₂ (250 x 4.6 mm, 5μm dp). Pressure 125 atm, 80°C, 2 mL/min. Modifier program: 98/2% CO₂/MeOH hold for 3 min. and then increased to 90/10 CO₂/MeOH at rate of 0.4%/min.

5 Figure 27 SFC separation of kavalactone extract. Column NH₂ (250 x 4.6 mm, 5μm dp). Pressure 125 atm, 60°C, 2 mL/min. Modifier program: 93/7% CO₂/MeOH for 3 min. and then increased to 90/10% CO₂/MeOH at rate of 0.2%/min.

10 Figure 28 SFC separation of kavalactone extract. Column protein C4 (250 x 4.6 mm, 5μm dp). Pressure 125 atm, 100°C, 2mL/min. Modifier program: 98.5/1.5% CO₂/MeOH for 2 min. and then increased to 90/10% CO₂/MeOH at rate of 0.2%/min.

15 Figure 29 SFC separation of kavalactone extract. Column protein C4 (250 x 4.6 mm, 5μm dp). Pressure 125 atm, 80°C, 2.5 mL/min. Modifier program: 98/2% CO₂/MeOH containing 0.1% isopropylamine for 3 min. and then increased to 90/10% CO₂/MeOH at rate of 0.4%/min.

20 Figure 30 SFC separation of kavalactone extract. Column diphenyl (250 x 4.6 mm, 5μm dp). Pressure: 125 atm for 3 min. and then increased to 195 atm at rate of 5 atm/min, 80°C, 2 mL/min. Modifier program: 98/2% CO₂/MeOH, then increased to 93/7% CO₂/MeOH at rate of 0.1%/min.

Figure 31 SFC separation of kavalactone extract. Column CN (250 x 4.6 mm, 5μm dp). Pressure 125 atm, 60°C, 2 mL/min. Modifier program: 98/2% CO₂/MeOH for 3 min. and then increased to 90/10% CO₂/MeOH at rate of 0.4%/min.

25 Figure 32 Semi-preparative SFC separation of kavalactone extract. column a single protein C4 (250 x 4.6 mm, 5μm dp). Pressure 125 atm, 80°C, 4 mL/min. Modifier program: 98/2% CO₂/MeOH containing 0.1% isopropylamine for 3 min. and then increased to 90/10% CO₂/MeOH at rate of 0.4%/min.

30 Figure 33 Semi-preparative SFC separation of kavalactone extract. Column two protein C4 (250 x 4.6 mm, 5μm dp) in series. Pressure 125 atm, 80°C, 4

mL/min. Modifier program: 98/2% CO₂/MeOH containing 0.1% isopropylamine for 3 min. increased to 90/10% CO₂/MeOH at rate of 0.4%/min. Injection volume - 50μL (100μg/μL).

Description of the Invention

As embodied and broadly described herein, the present invention is directed to methods for isolating and purifying bioactive substances from various natural sources. The invention is further directed to pharmaceutical preparations and dietary supplements which may be prepared with the bioactive substances and use of such pharmaceutical preparations and dietary supplements to treat various human ailments.

1. Supercritical Fluid Extraction

When great amounts of pressure are exerted onto a gas, the gas changes state to become a liquid. Above a certain pressure (the critical pressure) and temperature (the critical temperature), however, a gas may be pressurized further without liquifying. This combination of pressure and temperature is known as the critical point, and above it the gas becomes a supercritical fluid. A gas in the supercritical fluid state exhibits the diffusivity of a gas but has the solvating power of a liquid. The supercritical fluid may be pressurized to achieve densities close to 1.0 kg/l, similar to many liquids. A further property of supercritical fluids is that for a given solute, solvating power is a complex function of fluid density. Consequently, supercritical fluids are often used to selectively extract or separate specific compounds from a mixture by varying fluid density through changes in pressure and temperature.

Carbon dioxide is a commonly used volatile substance for supercritical fluid extractions. At temperatures of 39°C and above (its critical temperature) and at pressures between 200 and 600 bar, CO₂ is capable of removing caffeine from coffee and tea, some fragrances and flavor oils from certain plants and spices (U.S. Pat. Nos. 5,512,285 and 5,120,558), and some pharmacological active principles from certain plants and herbs. Depending on the temperature and pressure used, whether the temperature and pressure are varied during extraction, and the extraction method, different substances can be selectively removed or isolated from a plant species using supercritical fluid extraction.

In one embodiment of the present invention, CO₂ supercritical fluid extraction is used to extract bioactive substances from various natural sources including, but not limited to *Piper methysticum*, *Byrsonima* species, *Aesculus californica*, *Crataegus mexicana*, *Simmondsia chinensis*, *Pfaffia* species, *Bursera* species, *Turnera* species, and
5 *Heimia salicifolia*, *Psidium* species, *Enterlobium* species, *Ptychopetalum olacoides*, *Liriosma ovata*, and *Chaunochiton kappleri*.

Supercritical fluid extraction can be applied to a quantity of the root, leaf, bark, or any other part of a plant or herb, or combinations thereof, containing bioactive substances. Generally the specific part or parts are ground to form a powder or paste.
10 The powder or paste may be extracted with CO₂ at one or more temperatures, preferably a minimum of 45°C, and at least two pressures, preferably a minimum pressure between 200 and 400 bar and a maximum pressure between 400 and 600 bar. Use of more than one pressure, and more than one temperature, during extraction permits extraction of various bioactive substances which may be soluble in the CO₂ at specific pressure and
15 temperature levels.

In a further embodiment, the extraction may be performed with a mixture of CO₂ and at least one other volatile substance such as butane, propane, ethanol, hexane, or any other appropriate volatile substance known to those of skill in the art. The gases may be used at any optimum ratio relative to one another. In a preferred embodiment,
20 the extraction is performed with a combination of CO₂ and ethanol in a ratio of 17:3.

In another embodiment, the plant or herb may be crushed, macerated, or mixed with a solvent and the solvated mixture may then be extracted with supercritical fluid CO₂. See for example U.S. Pat. No. 4,985,265. Under the heavy pressures of supercritical fluid extraction, the CO₂-cosolvent mixture remains in the liquid monophasic
25 state. This type of liquid-liquid extraction improves elution of certain analytes. A wide variety of solvents appropriate for solvating various bioactive substances in natural sources may be used including, but not limited to, alcohols, weak acids, ketones, chloro derivatives, hydrocarbons, fluorinated hydrocarbons, acetates, ethers, or combinations thereof.

The extraction of the present invention is carried out for a minimum of 5 minutes, preferably at least 30 minutes and more preferably 60 minutes, during which extracted analytes are collected in a collection receptacle, preferably a solid phase trap packed with C18. After completion of extraction, the trap may be rinsed with a solvent
5 appropriate for solvating the bioactive substances that have been extracted, such as, for example, 50/50 ethanol/methylene chloride for kavalactones, to collect most of the analytes in the trap. Similar methods of the present invention are outlined in Example 1 and Example 2 below wherein seven different kavalactones were extracted from a Kava root. The methods of the present invention can be used to extract bioactive
10 substances from one natural source at a time, or from multiple natural sources in one extraction.

A supercritical fluid extraction of the present invention can be performed as a batch extraction, as a continuous cascading extraction, as a countercurrent solvent extraction, or a combination thereof. The majority of supercritical fluid extractions in
15 the field of natural products has involved configurations of equipment which are batch loaded systems. In these systems, extraction vessels are loaded with raw material, sealed, and the pressure and temperature increased to the desired supercritical processing range. After extraction is completed, the pressure and temperature are decreased, the vessel opened, and the spent natural source removed before the process
20 can be repeated. To date, this process has not proven to be economically viable except in instances where it is performed at sufficiently large scale (e.g. the decaffeination of coffee) or the target compound is of sufficiently high value. In a continuous cascading extraction, multiple extraction vessels are sequentially entered on-line in a continuous manner, with the supercritical fluid passing from vessel to vessel, collecting specific
25 targeted compounds in each vessel. See U.S. Pat. No. 5,120,558; see also Stahle, et al., Dense Gases for Extraction and Refining, Springer-Verlag, Berlin, 1988. This method is advantageous in that the average loading rate of the CO₂ is increased because the CO₂ fluid carrying low quantities of analyte from partially extracted vessels can dissolve more analyte from the new vessel sequentially introduced, thus effectively increasing the
30 average loading rate of the CO₂ fluid, and hence, the analyte extraction rate per hour.

In a countercurrent extraction process of the present invention, bioactive substances from plants and herbs are extracted and concentrated in a series of countercurrent mechanical presses. See U.S. Pat. No. 6,013,304. The presses may be kept at high pressure and escalated temperature as outlined above to facilitate the supercritical fluid extraction. As the supercritical fluid becomes more highly concentrated in its analyte content through sequential pressing, the analyte containing fluid is recirculated to the first press to extract more analyte and become more concentrated.

2. Dense Gas Extraction

Due to its non-flammable nature, as opposed to propane or butane, and excellent solvating properties for a wide range of target analytes, CO₂ has become the most common volatile substance used in the art of supercritical fluid extraction. However, CO₂ is effective as an extraction medium only at extreme pressures. This results in a high cost of equipment to perform the extraction and to inherent dangers associated with extreme pressure vessels. Furthermore, the cost of scaling up such equipment is prohibitive so the equipment tends to remain small scale. Additionally, supercritical CO₂ extraction systems operate at temperatures in excess of 39°C. Holding labile natural materials at such temperatures for long periods during processing may result in thermally or enzymatically induced spoilage.

Recently, non-chlorinated fluorocarbon solvents have been disclosed for extracting fragrances and flavors from natural materials. See U.S. Pat. No. 5,512,285. In one embodiment of the present invention, non-chlorinated fluorocarbon solvents including, but not limited to, trifluoromethane, difluoromethane, fluoromethane, pentafluoroethane, 1,1,1-trifluoroethane, 1,1-difluoroethane, 1,1,1,2,2,3,3-heptafluoropropane, 1,1,1,3,3,3-hexafluoropropane, 1,1,1,2,2-pentafluoropropane, 1,1,1,2,2,3-hexafluoropropane, 1,1,2,2,3,3-hexafluoropropane, 1,1,1,2,3,3-hexafluoropropane, and 1,1,1,2-tetrafluoroethane may be used. The solvent used in the present invention may be a mixture of these solvents to tailor the boiling point of the mixture to a particular process and facilitate the selective elution of specific bioactive substances. The solvent may be further modified by mixing with another volatile substance such as butane,

hexane, ethanol or any other appropriate substance. In a preferred embodiment, the non-fluorocarbon solvent used for extraction is a tetrafluoroethane, preferably 1,1,1,2-tetrafluoroethane. In a further preferred embodiment, the tetrafluoroethane is unmodified.

5 In a process of the present invention, ground or crushed natural sources such as plants and/or herbs are contacted with a non-chlorinated fluorocarbon solvent in the liquid phase so as to charge the solvent with analyte. Charged solvent is collected and removed to isolate the analyte. In one embodiment of the present invention, the herb or plant material is contacted with a non-chlorinated fluorocarbon solvent in an extraction
10 vessel after the vessel has been sealed and air has been removed. The resulting mixture of the solvent and natural source is maintained under pressure so that the natural source and solvent are in intimate contact to charge the solvent with analyte. This type of extraction may be carried out in any extractor which may be sealed and evacuated of air as required. The extractor may be made of stainless steel, heavy walled glass, or any
15 other non-reactive material which is able to withstand elevated or reduced pressures. The extraction may be performed at any suitable temperature and is preferably carried out at or below room temperature.

 In another embodiment, the extraction may be carried out as a supercritical fluid extraction at increased pressures and varied temperatures. Particularly if the
20 fluorinated hydrocarbon solvent is modified with another volatile substance with a higher boiling point, increased pressures and temperatures may be used to properly elute desired analytes.

 In another embodiment, the plant or herb may be crushed, macerated, or mixed with a solvent and the solvated mixture may then be extracted with fluorinated
25 hydrocarbon solvents or modified fluorinated hydrocarbon solvents. This type of liquid-liquid extraction may improve elution of certain analytes. A wide variety of solvents appropriate for solvating various bioactive substances in natural sources may be used including, but not limited to, alcohols, weak acids, ketones, chloro derivatives, hydrocarbons, fluorinated hydrocarbons, acetates, ethers, or a combination thereof.

Extraction may be carried out batch-wise, as a continuous-cascading extraction, or as a countercurrent-solvent extraction. In a preferred embodiment of the present invention, bioactive substances are extracted from plants and/or herbs using fluorocarbon solvents in a continuous cascading extraction. The extractor may
5 communicate with an evaporator. During evaporation of the solvent from the eluted analyte, the solvent may be allowed to pass intermittently from the reactor to the evaporator to maintain a level of liquid and a gas-filled head space in the evaporator. Evaporation of the solvent may be achieved by withdrawal of gaseous solvent from the head space of the evaporator. The withdrawn gaseous solvent may be transferred to a
10 compressor or some similar device to reliquify the solvent, thereby economically reusing the solvent.

In another embodiment, the evaporator may have one or more sources of heat to control the temperature of the evaporator during evaporation of the solvent. In a further embodiment, the heat source may be thermostatically controlled to provide
15 constant evaporation temperature. The non-chlorinated fluorinated hydrocarbon solvents generally boil off before the desired analytes and it is therefore not necessary to elevate the temperature of distillation of the solution during the solvent recovery phase of the process. Extracts produced in this manner contain very low levels of solvent residues.

20 The vapor pressure of most fluorocarbon solvents is greater than atmospheric pressure at room temperature. For example, the vapor pressure of 1,1,1,2-tetrafluoroethane is 5.6 bar at 20°C. In a preferred embodiment, extraction is thus carried out at a pressure from 0-10 bar and preferably 3.5-6.0 bar. Although most of these solvents must be handled in equipment which is capable of tolerating such
25 pressures, this equipment is a fraction of the cost of equivalent equipment required for handling supercritical CO₂, and a fraction of the degree of sophistication or hazard inherent in a manufacturing plant for handling liquefied hydrocarbon gases under pressure.

3. Supercritical Fluid Chromatography

Once bioactive substances are collected as extracts from plants and herbs following any extraction method, the substances in the extracts can be separated and isolated using various techniques such as gas chromatography (GC) or high pressure liquid chromatography (HPLC). Gas chromatography, however, is not scalable to provide a method for isolation of each substituent in large quantity. Liquid chromatography, on the other hand, has the draw back of utilizing large volumes of solvent.

In one embodiment of the present invention, packed column supercritical fluid chromatography is used to separate the bioactive substances in extracts obtained from various natural sources. Bioactive substances that can be separated from such sources include terpenes, terpenoids, flavones and flavonoids, steroids, sterols, saponins and sapogenins, alkanes, alkaloids, amines, amino acids, aldehydes, alcohols, fatty acids, lipids, lignans, phenols, pyrones, butenolides, lactones, chalcones, ketones, benzenes, cyclohexanes, glucosides, glycosides, cyanidins, furans, phorbols, quinones and phloroglucinols. The invention can also be applied to the recovery of bioactive substances that are large molecular weight materials such as proteins, peptides, enzymes, polysaccharides and carbohydrates. Sources from which bioactive substances can be isolated include, but are not limited to various plant species of Kava (such as Kava root), Byrsonima, Aesculus (e.g. *A. californica*), Crataegus mexicana, Jojoba, Pfaffia, Alternanthera (e.g. *A. repens*), Bursera, Turnera, Perezia, Heimia (e.g. *H. salicifolia*), Psidium, Enterlobium, Ptychopetalum (e.g. *P. olacoides*), Liriosma (e.g. *L. ovata*) and Chaunochiton (e.g. *C. kappleri*). Plants from which extracts can be prepared and natural substances isolated according to the invention include the higher plants: Acanthopanax, Acanthopsis, Acanthosicyos, Acanthus, Achyranthes, Acokanthera, Aconitum, Acorus, Acronychia, Actaea, Actinidia, Adenia, Adhatoda, Aegle, Aesculus, Aframomum, Agastache, Agathosma, Alchemilla, Aleurites, Allium, Aloe, Alonsoa, Aloysia, Alphonsea, Alpinia, Alternanthera, Amaranthus, Amomum, Amphipterygium, Amyris, Anchusa, Ancistrocladus, Anemopsis, Angelica, Annona, Anonidium, Anthemis, Antidesma, Apium, Aralia, Aristolochia, Artemisia, Artocarpus, Asarum, Asclepias, Asimina, Aspalanthus, Asparagus, Aspidosperma, Astragalus, Astronium, Atropa,

- Avena, Azadirachta, Azara, Baccharis, Bacopa, Balanites, Bambusa, Barleria, Barosma, Bauhinia, Belamcanda, Benincasa, Berberis, Berchemia, Bixa, Bocconia, Borago, Boronia, Boswellia, Brosimum, Brucea, Brunfelsia, Bryonia, Buddlejia, Bulnesia, Bupleurum, Bursera, Byrsonima, Calamintha, Calea, Calophyllum, Camellia,
- 5 Camptotheca, Cananga, Canarium, Canella, Capparis, Capsicum, Carthamus, Carum, Cassia, Cassine, Castanospermum, Catalpa, Catha, Catharanthus, Cayaponia, Cecropia, Centaurea, Centipeda, Centranthus, Cephaelis, Chiranthodendron, Chondrodendron, Chrysophyllum, Cimicifuga, Cinchona, Cinnamomum, Cistus, Citrus, Clausena, Cnicus, Coccoloba, Codonopsis, Coffea, Coix, Cola, Coleus, Colletia, Combretum,
- 10 Commiphora, Cordia, Coriaria, Correa, Corydalis, Costus, Crataegus, Croton, Cryptolepis, Cudrania, Cuminum, Cuphea, Cucurma, Cyclanthera, Cymbopogon, Cynara, Cynoglossum, Cyperus, Cyrtocarpa, Dalbergia, Dalea, Danae, Daphne, Datura, Daucus, Decadon, Dendrocalamus, Dendropanax, Deppea, Derris, Desmos, Dichrostachys, Dictamnus, Digitalis, Dillenia, Dioscorea, Dioscoreophyllum, Diosma,
- 15 Diospyros, Drimys, Duboisia, Duguetia, Dysoxylum, Echinacea, Eclipta, Ehretia, Ekebergia, Eleagnus, Elettaria, Eleutherococcus, Encelia, Entandrophragma, Ephedra, Epimedium, Eriobotrya, Erodium, Eryngium, Erythrochiton, Erythroxylum, Escholzia, Esenbeckia, Euclea, Eucommia, Euodia, Eupatorium, Fabiana, Ferula, Fevillea, Fittonia, Flindersia, Foeniculum, Galesia, Galphimia, Garcinia, Gaudichaudia, Gaultheria,
- 20 Gelsemium, Gentiana, Geranium, Gigantochloa, Ginkgo, Glochidion, Gloeospermum, Grewia, Greyia, Guaiacum, Gymnema, Haematoxylum, Hamamelis, Hamelia, Harpagophytum, Hauya, Heimia, Helleborus, Hieracium, Hierochloa, Hilleria, Hippophae, Houttuynia, Hovenia, Humulus, Huperzia, Hura, Hybanthus, Hydnocarpus, Hydnophytum, Hydrastis, Hydrocotyle, Hymenaea, Hyoscamus, Hypericum, Hyptis,
- 25 Hyssopus, Iboza, Idiospermum, Ilex, Illicium, Indigofera, Inga, Inula, Iochroma, Iresine, Iris, Jacaranda, Jatropha, Juniperus, Justicia, Kadsura, Kaempferia, Lactuca, Lagochilus, Larrea, Laurus, Lavandula, Lawsonia, Leonurus, Leucas, Ligusticum, Lindera, Lippia, Liriosma, Litsea, Lobelia, Lonchocarpus, Lonicera, Lycium, Macfadyena, Maclura, Mangifera, Mansoa, Marcgravia, Marrubium, Martinella, Matricaria, Maytenus,
- 30 Medicago, Melissa, Mentha, Mimosa, Mimusops, Mitragyna, Montanoa, Morkillia,

Mouriri, Mucuna, Mutisia, Myrica, Myristica, Nardostachys, Nepeta, Nicotiana, Ocotea, Olea, Oncoba, Ophiopogon, Origanum, Pachyrhizus, Panax, Papaver, Pappea, Parthenium, Passiflora, Paullinia, Pelargonium, Penstemon, Perezia, Perilla, Persea, Petiveria, Petroselinum, Peucedanum, Peumus, Pfaffia, Phoebe, Phyllanthus, Phytolacca, Pilocarpus, Pimenta, Pimpinella, Pinellia, Piper, Piqueria, Pithecellobium, Pittosporum, Plectranthus, Pleuropetalum, Podophyllum, Pogostemon, Polygala, Polygonum, Polymnia, Psacalium, Psychotria, Pterygota, Ptychopetalum, Pueraria, Punica, Pycnanthemum, Pygeum, Quararibea, Quassia, Quillaja, Randia, Ratibida, Rauvolfia, Rehmannia, Renealmia, Rheum, Rollinia, Rorippa, Rosmarinus, Rudbeckia, Ruellia, Rumex, Ruscus, Ruta, Saccharum, Salix, Salvia, Sambucus, Sanguinaria, Sapium, Sassafras, Satureja, Sceletium, Schizandra, Securidaca, Securinega, Serenoa, Simmondsia, Smilax, Stachytarpheta, Stachys, Staurogyne, Stelechocarpus, Stephania, Sterculia, Stevia, Strophanthus, Strychnos, Symphytum, Syzygium, Tabebuia, Tabernaemontana, Tabernanthe, Tanacetum, Taxus, Tecoma, Terminalia, Teucrium, Thaumtococcus, Tribulus, Trifolium, Trigonella, Triplaris, Triumfetta, Turnera, Tussilago, Tylophora, Tynnanthus, Uncaria, Urginea, Urtica, Uvaria, Vaccinium, Valeriana, Vallesia, Vangueria, Vanilla, Vellozia, Vepris, Verbascum, Verbena, Vetiveria, Virola, Viscum, Vismia, Vitex, Voacanga, Warburgia, Withania, Zanthoxylum, Zingiber, Zizyphus and Zygophyllum. In addition to the genera of higher plants listed above, compounds can be recovered from such biological sources as algae, bacteria, fungi, lichens, mosses, and marine organisms such as corals, sponges, tunicates or other invertebrate or vertebrate organisms.

A variety of stationary phases, pressures, temperatures, and modifier concentrations can be applied to optimize the separation. Separations of extracted kavalactones are used to illustrate some methods of packed column supercritical fluid chromatography separation of the present invention in Examples 3 to 10 below. This invention is significant given the amenability of SFC for both semi-preparative and preparative scale fractionations which could ultimately afford isolation of each substituent in an analyte mixture in milligram quantities. Furthermore, according to the present invention, equipment for the separation of analytes can be built to communicate

with extraction and evaporation equipment to allow a continuous assembly line process for extracting, separating, and isolating specific bioactive substances from selected plants and herbs.

4. Therapeutic Plant Extracts and Their Uses

5 i) Extracts of Byrsonima

In one embodiment of the present invention, extracts of Byrsonima species, such as Byrsonima crassifolia, comprising a variety of triterpenes, amino acids, and/or flavonoids are prepared. These extracts may be prepared using water, non-aqueous solvents such as methanol, ethanol, or ethyl acetate, a mix of water with a non-aqueous solvent, or using one of the extraction methods described above. The extracts of Byrsonima may be administered to humans in therapeutic quantities to treat a variety of ailments including, but not limited to, gastrointestinal disorders (e.g. diarrhea, Chron's disease, irritable bowel syndrom), and neurological and vascular disorders such as stroke, Parkinson's disease, and Alzheimer's disease.

15 The extracts of Byrsonima may further be combined with extracts from other plant species including, but not limited to, Psidium species such as Psidium Guajava, and Enterolobium species such as Enterolobium cyclocarpum. The extracts of these other species may be prepared by any method known in the art or any of the methods described above.

20 In a further embodiment of the invention, the extracts of the Byrsonima species and/or any other extracts to be combined with the Byrsonima extract, may be separated to isolate specific bioactive substances to treat specific ailments. For example, pipecolic acid may be isolated from the extracts of the leaves and bark of Byrsonima crassifolia. The separation may be performed using any separation technique known to those of skill in the art, or a packed column supercritical fluid chromatography separation method as described herein. Once separated, the pipecolic acid may be administered by itself, or in combination with other bioactive substances from Byrsonima or other plant extracts, in therapeutic quantities to treat neurological and vascular disorders.

In still a further embodiment, extracts of the *Byrsonima* species alone or in combination with extracts from other plant or herb species, or isolated bioactive substances of the *Byrsonima* species alone or in combination with bioactive substance of other plants or herbs, may be made into a capsule, pill, pastille, or elixir, in combination with other inert or pharmacological ingredients to be administered to patients.

ii) Extracts of North American Varieties of *Aesculus* and *Crataegus* Species

In one embodiment of the present invention, extracts of *Aesculus* species, such as *Aesculus californica* comprising aescin and a variety of triterpene glycosides, and *Crataegus* species, such as *Crataegus mexicana* comprising a variety of flavonoids and oligomeric procyanidins are prepared. These extracts may be prepared using water, non-aqueous solvents such as methanol, ethanol, or ethyl acetate, a mix of water with a non-aqueous solvent, or using one of the extraction methods described above. The extracts of *Aesculus californica* and *Crataegus mexicana* may be administered to humans, either each on their own or in combination, in therapeutic quantities to treat a variety of ailments including, but not limited to, cardiac and vascular disorders. These extracts may also be given as a dietary supplement to provide a cardio and vascular protective effect, particularly in case of cardiac ischemia and life-threatening reperfusion-induced cardiovascular lesions. See U.S. Pat No. 5,925,355.

The extracts of *Aesculus californica* and *Crataegus mexicana* may further be combined with extracts from other plant species including, but not limited to, *Bursera* species, such as *Bursera microphylla*. The extracts of these other species may be prepared by any method known in the art or any of the methods described above.

In a further embodiment of the invention, the extracts of the *Aesculus californica* and *Crataegus mexicana* species and/or any other extracts to be mixed with either or both of the *Aesculus* and *Crataegus* extracts, may be separated to isolate specific bioactive substances to treat specific ailments. For example, hydroquinone may be isolated from the extracts of *Aesculus californica*. The separation may be performed using any separation technique known to those of skill in the art, or a packed column supercritical fluid chromatography separation method as described herein. Once

separated, the hydroquinone acid may be administered by itself, or in combination with other bioactive substances from *Aesculus californica*, *Crataegus mexicana*, or other plant extracts, in therapeutic quantities to treat cardiac and vascular disorders.

In still a further embodiment, extracts of the *Aesculus* and *Crataegus* species alone or in combination with extracts from other plant or herb species, or isolated bioactive substances of the *Aesculus* and *Crataegus* species alone or in combination with bioactive substance of other plants or herbs, may be made into a capsule, pill, pastille, or elixir, in combination with other inert or pharmacological ingredients to be administered to patients.

iii) Extracts of Jojoba

In one embodiment of the present invention, extracts of *Simmondsia chinensis* (also known as *S. californica* and Jojoba), particularly extracts of defatted Jojoba meal, comprising simmondsin are prepared using water, non-aqueous solvents such as methanol, ethanol, or ethyl acetate, a mix of water with a non-aqueous solvent, water and non-aqueous solvents in sequence, or using one of the extraction methods described above. The extracts of *Simmondsia* may be administered to humans in therapeutic quantities as hunger satiation and weight reduction agents.

Extracts of *Simmondsia* may further be combined with extracts from other plant species. The extracts of the other species may be prepared by any method known in the art or any of the methods described above. In a preferred embodiment, simmondsin is separated from other substances in the *Simmondsia* extract. The separation may be performed using any separation technique known to those of skill in the art, or a packed column supercritical fluid chromatography separation method as described herein. Once separated, simmondsin may be administered by itself, or in combination with other bioactive substances from *Simmondsia* or other plant extracts, in therapeutic quantities as a hunger satiation or weight reduction agent. Therapeutically effective amount to satiate hunger are between 5 and 500 mg/kg body weight, preferably between 10 and 250 mg/kg body weight, more preferably between 20 and 100 mg/kg body weight and even more preferably between 25 and 50 mg/kg body weight.

In still a further embodiment, extracts of *Simmondsia* alone or in combination with extracts from other plant or herb species, or isolated simmondsin alone or in combination with bioactive substances of other plants or herbs, may be made into a capsule, pill, pastille, or elixir, in combination with other inert or pharmacological ingredients to be administered to patients.

iv) Extracts of *Turnera* and *Pfaffia* Species

In one embodiment of the present invention, extracts of *Turnera* species, such as *Turnera diffusa*, and *Pfaffia* species, such as *Pfaffia paniculata*, comprising various terpenes and phytochemicals are prepared. These extracts may be prepared using water, non-aqueous solvents such as methanol, ethanol, or ethyl acetate, a mix of water with a non-aqueous solvent, or using one of the extraction methods described above. The extracts of *Turnera* species and *Pfaffia* species may be administered to humans, either each alone or in combination with one another, in therapeutic quantities to treat a variety of ailments including, but not limited to, diabetes, rheumatism, ulcers, various cancers such as leukemia, chronic coughing, nephritis, orchitis, and spermatorrhea. These extracts may also be administered as a dietary supplement or health tonic to increase sexual drive, aid digestion, and increase fertility.

Extracts of *Turnera* and *Pfaffia* species may further be combined with extracts from other plant species including, but not limited to *Muirapapuama* (a crude drug derived from various species including *Ptychopetalum olacoides*, *Liriosma ovata* and *Chaenochiton kappleri*). The extracts of these other species may be prepared by any method known in the art or any of the methods described above.

In a further embodiment of the invention, the extracts of the *Turnera* and *Pfaffia* species and/or any other extracts to be mixed with the *Turnera* and/or *Pfaffia* extracts, may be separated to isolate specific bioactive substances to treat specific ailments. For example, β -sitosterol may be isolated from the extracts of *Turnera diffusa* and/or *Pfaffia paniculata*. The separation may be performed using any separation technique known to those of skill in the art, or a packed column supercritical fluid chromatography separation method as described herein. Once separated, the β -sitosterol may be administered by itself, or in combination with other bioactive substances from

Turnera, Pfaffia, or other plant extracts, in therapeutic quantities as a health tonic to support either or both, male and/or female sexual function.

In still a further embodiment, extracts of the Turnera and Pfaffia species alone or in combination with extracts from other plant or herb species, or isolated bioactive substances of the Turnera and Pfaffia species alone or in combination with bioactive substance of other plants or herbs, may be made into a capsule, pill, pastille, or elixir, in combination with other inert or pharmacological ingredients to be administered to patients.

v) Extracts of Heimia Species

In one embodiment of the present invention, extracts of Heimia species, such as Heimia solicifolia, comprising a variety of alkaloids and quinones are prepared. These extracts may be prepared using water, non-aqueous solvents such as methanol and ethanol, a mix of water with a non-aqueous solvent, or using one of the extraction methods described above. The extracts of Heimia species may be administered to humans, either each alone or in combination, in therapeutic quantities to treat a variety of ailments including, but not limited to, joint and muscle inflammation. In a preferred embodiment, extracts of Heimia species are combined and administered in therapeutic quantities as a non-steroidal anti-inflammatory (NSAID).

Extracts of Heimia species may further be combined with extracts from other plant species. The extracts of these other species may be prepared by any method known in the art or any of the methods described above.

In a further embodiment of the invention, the extracts of a Heimia species and/or any other extracts to be mixed with other Heimia extracts, may be separated to isolate specific bioactive substances to treat specific ailments. For example, the alkaloids cryogenine and nesodine may be isolated from Heimia salicifolia. The separation may be performed using any separation technique known to those of skill in the art, or the packed column supercritical fluid chromatography separation method described herein. Once separated, cryogenine and nesodine may be administered by themselves, in combination, or in combination with other bioactive substances from Heimia salicifolia,

or other plant extracts, in therapeutic quantities to treat inflammation of joints, muscles, or other tissue.

In still a further embodiment, extracts of the *Heimia* species alone or combined with extracts from other plant or herb species, or isolated bioactive substances of the *Heimia* species also alone or combined with bioactive substance of other plants or herbs, may be made into a capsule, pill, pastille, or elixir, in combination with other inert or pharmacological ingredients to be administered to patients.

The following experiments are offered to illustrate embodiments of the invention, and should not be viewed as limiting the scope of the invention.

10 Examples

Example 1 Supercritical Fluid Extraction - CO₂ Extraction

The kavalactone supercritical fluid extractions (SFE) described in Examples 1-2 were performed using a 3 mL extraction vessel. Each extraction contained 0.5 grams of finely ground Kava root. Various experimental conditions, as described below, were used to determine the conditions that maximized recovery of kavalactones. The SFE procedures were performed for 60 minutes at a flow rate of 2 mL/min of liquid CO₂.

Supercritical fluid extractions were performed at 350 atm and 450 atm. A solid phase trap packed with C18 was used to collect the extracted analytes. The trap temperature was set at +10°C. After completing each supercritical fluid extraction, the trap was rinsed with 10 mL of 50/50% mixture of ethanol/CH₂C₁₂. The extract volume was then adjusted to 25 mL using CH₂C₁₂.

Kavalactone Standards - Because no pure kavalactone standards were available, the SFE extracts were compared with Kava root extracts obtained by conventional sonication methods. For this purpose 0.5 gram of Kava root was sonicated for 30 minutes in 25 mL of 50/50 CH₂C₁₂/MeOH as an extraction solvent. The extract was then filtered through a 2 µm filter paper. The extract was then analyzed with a Hewlett Packard Model 5890 Gas Chromatograph coupled to a Hewlett Packard Model 5972 Mass Spectrometer. Extract from sonication was assumed to yield a 100%

recovery of all kavalactones in the root sample. Kavalactone recovery using CO₂ SFE was compared with the kavalactone recover using CH₂Cl₂/MeOH sonication.

Columns 1 and 2 of Table 1 shows SFE recoveries of different kavalactones from Kava root using different SFE conditions. The recoveries are expressed as a percentage of the recovery obtained by conventional sonication methods. Peak identification were obtained by comparison of three most intense ions in mass spectrum of each peak and those reported by Viorica Lopez-Avila et al. V. Lopez-Avila and Benedicto, J. High Resolut. Chromatogr., 20, 555 (1997).

Table 1
Percent Recovery of Different Kavalactones from
Kava Root Using Supercritical Fluid Extraction*

Compound	350 atm, 60°C		450 atm	
	60°C 100% CO ₂	60°C 100% CO ₂	60°C 85% CO ₂ 15% EtOH	60°C 85% CO ₂ 15% EtOH
7,8-Dihydrokavain	92.9% (7)	97.5% (6)	92.7% (2)	91.1% (5)
Kawain	93.6% (5)	100.0% (4)	102.9% (4)	107.0% (4)
5,6-Dihydrokavain	86.1% (8)	80.3% (5)	74.0% (8)	79.9% (7)
5,6,7,8-Tetra- hydroangonin	97.9% (5)	92.9% (8)	96.4% (4)	106.0% (6)
Dihydromethysticin	93.2% (8)	88.4% (6)	95.3% (8)	104.1% (4)
Yangonin	84.7% (11)	67.6% (12)	72.5% (9)	84.1% (9)
Methysticin	95.9% (7)	66.2% (10)	111.1% (7)	137.6% (12)

* = % recovery are based on comparison of SFE with 50/50 CH₂Cl₂/MeOH sonication extraction.

() = % RSD for three replicate extractions.

Example 2 CO₂ Extractions with Ethanol

Another supercritical fluid chromatography extraction was performed to test the efficiency of obtaining kavalactones using a mixture of CO₂ and ethanol. In this experiment an extraction solution of 85% CO₂ and 15% ethanol was used at 350 atm and 450 atm of pressure. The trap temperature was held at 60°C. Table 1 shows that some species of kavalactones, notably Kavain and Methysticin were more efficiently extracted from SFE than with sonication. Table 2 shows the retention times, molecular

weights (MW), and three most intense ions in the mass spectra analysis of kavalactones isolated through SFE, as described above.

Table 2
Three Most Intense Ions Found in the Electron Impact Mass Spectra
of Kavalactones Root Extract via SFE

No.	Compound Name	Retention Time	MW	Three most intense ions (m/z)
1	7,8-Dihydrokavain	27.85	232	127 (100), 91, 117
2	Kavain	29.05	230	98 (100), 68, 69
3	5,6-Dihydrokavain	29.85	228	228 (100), 157, 69
4	5,6,7,8-Tetrahydro angonin	30.71	262	121 (100), 147, 262
5	Dihydromethysticin	31.96	276	135 (100), 276, 136
6	Yanagonin	32.95	258	258 (100), 187, 230
7	Methysticin	33.16	274	148 (100), 135, 274

Figure 1 shows the Gas Chromatograph/Mass Spectroscopy (GC/MS) separation of kavalactones extracted using SFE. Figures 2-8 show mass spectra of each kavalactone listed in Table 2. Table 3 shows retention times and four most intense ions for the mass spectra of other peaks that eluted earlier than the major kavalactones.

Table 3
Three Most Intense Ions Found in the Electron Impact Mass Spectra
of Peaks Eluted Earlier than the Major Kavalactones Extracted via SFE

No.	Compound Name	Retention time	Four most intense ions (m/z)
1	Unknown	19.40	91 (100), 65, 188, 97
2	Unknown	22.01	186 (100), 95, 128, 155
3	Unknown	23.01	121 (100), 218, 77, 78
4	Unknown	24.35	135 (100), 77, 232, 136
5	Unknown	26.93	135 (100), 230, 115, 128

Figures 9-13 show spectra of other major peaks which eluted before kavalactones (tR = 19.40, 22.1, 23.01, 24.35, and 26.93 min). It is believed that the peak with tR-23.1 is a spike (ghost peak) which appeared in MS. Figure 14 shows GC/MS chromatogram of kavalactones extracted via sonication.

To compare the absolute weights of extracts obtained by SFE and sonication, 0.5 grams of Kava root was extracted via both SFE and sonication, as discussed above. Each extract was then transferred to a vial of known weight. The solvent in each extract was evaporated under a stream of nitrogen. Table 4 shows the weight and percent of

extracted analytes from 0.5 gram of Kava root using both an SFE and sonication technique.

Table 4
Extracted from Kava root via
Supercritical Fluid Extraction and Solid-Liquid Extraction (Sonication)

	Sample weight extraction (g)	Weight of extract (g)	Percent weight of analyte extracted
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5	SFE, 350 atm, 60°C 85/15 CO ₂ /EtOH		
10	2 mL/min	1.0	0.0716
	SFE, 350 atm, 60°C 85/15 CO ₂ /EtOH		
	2 mL/min	0.5	0.038
15	Sonication, 15 mL 50/50 CH ₂ Cl ₂ /MeOH for 30 min	0.5	0.039
			7.16
			7.6
			7.8

These results show that more than 90% of the measured kavalactones can be extracted via pure CO₂, however, a more complete extraction of kavalactones was found using a composition of 85% CO₂ and 15% ethanol as the supercritical fluid.

Example 3 Separation of Kavalactones Using Supercritical Fluid Chromatography

The following separations were performed using a Hewlett Packard Model G1205A supercritical fluid chromatograph (SFC) system equipped with a variable UV detector. The detection wavelength was set to 254 nm. Different columns and chromatography conditions were applied in order to determine the most advantageous separation of kavalactones.

Figures 15-17 show results of experiments wherein kavalactone extracts were subjected to SFC using NH₂, DIOL, and CN columns. The chromatography conditions for the NH₂ column was: Column Material: NH₂ with water; Brand Name: Altec Sphenosorb; Length: 25cm; Inner Diameter: 4.6mm ID; Pressure: 125 atm; Temperature: 60°C; Flow Rate: 2 mL/min liquid CO₂.

The modifier programming started with 2/98% MeOH/CO₂ hold for 3 min., and then increased to 10/90% MeOH/CO₂ at a rate of 0.4% min. The chromatography conditions for the DIOL column was: Column Material: DIOL; Brand Name: Vydac

Model Supleco; Length: 25cm; Inner Diameter: 4.6mm ID; Pressure: 125 atm; Temperature: 60°C; Flow Rate: 2 mL/min liquid CO₂.

Modifier programming started with 2/98% MeOH/CO₂ hold for 3 min., and then increased to 10/90% MeOH/CO₂ at a rate of 0.4% min. The chromatography conditions for the Cyano (CN) column was: Column Material: CN; Brand Name: Altec; Length: 25cm; Inner Diameter: 4.6mm ID; Pressure: 275 atm; Temperature: 60°C; Flow Rate: 2 mL/min liquid CO₂. Modifier programming started with 2/98% MeOH/CO₂ at a rate of 0.4% min.

As can be seen upon reference to Figures 15-17, the best kavalactone separations were obtained with the NH₂ column. Both the CN and DIOL column provided some separation, however co-elution of components was observed.

Example 4 Optimization of CN Column Conditions

Figure 18 shows the results of an experiment wherein kavalactone SFE extracts were separated with SFC at a higher temperature (80°C) using the CN column. All other chromatography conditions were the same as described for CN above. Selectivity of the column at 80°C was changed in that some of the lactones were separated which had co-eluted at 60°C. In addition, some lactones which co-eluted at 80°C were separated previously at 60°C.

Separation of kavalactones did not improve at a lower temperature (40°C) using a CN column under the same conditions. Additionally, a change in modifier concentration (modifier programming start with 2/98% MeOH/CO₂ hold for 3 min., and then increased to 10/90% MeOH/CO₂ at rate of 0.5%/min.) and pressure (125 atm) did not change the selectivity of the column as shown in Figure 19.

Example 5 Optimization of NH₂ Column Conditions

The SFC separation of kavalactone SFE extracts were then optimized with an NH₂ chromatography column under varying conditions. Figures 20 and 21 show separation of kavalactone extracts on NH₂ columns at 40°C and 80°C, respectively. The chromatography conditions were: Pressure 125 atm, and flow rate of 2 mL/min liquid CO₂. Modifier programming started with 2/98% MeOH/CO₂ hold for 3 min., and then increased to 10/90% MeOH/CO₂ at a rate of 0.4%/min. As shown in Figure 20, the

lower temperature separations decreased column selectivity and lactones co-eluted. At higher temperatures, a better solution was obtained for components eluted at 23.39 and 23.97 minutes (Figure 21) compared to the separation obtained at 60°C (Figure 15).

Figure 22 shows the results of a separation of the same Kava root extract on an NH₂ column, using the same conditions as described with Figure 21, with the exception that pressure was increased to 275 atm. Co-elution of several components was observed. The modifier concentration was then varied to optimize the elution time of the analytes. For this experiment the same pressure (125 atm), temperature (80°C), flow (2mL/min of liquid CO₂), and column (NH₂) was used with the exception that the modifier programming started with 7/93% MeOH/CO₂ hold for 3 minutes and then increased to 10/90% CO₂/MeOH at the rate of 0.2% minutes (Figure 23). A similar separation as Figure 21 was obtained. However, the analysis time was reduced from 24 minutes to 11 minutes.

The 7 peaks which were obtained using the NH₂ column are the kavalactones that were identified using the supercritical fluid extraction of Kava root and GC/MS. The area percentage of each peak in the chromatogram was as follows: 3, 9.5, 50, 6.9, 5.8, 4.0 and 20%.

Example 6 SFE of Kavalactones with CO₂ and 15% ethanol-modified CO₂.

All supercritical fluid extractions described in the Examples were performed using an Isco-Suprex (Lincoln, NE) Prepmaster equipped with an ACCUTRAP™ and variable flow restrictor. In each extraction 0.5 gram of Kava root, which was previously ground, was used. Extractions were performed for 60 minutes at a flow rate of 2 mL/min of liquid CO₂. Two pressures (350 and 450 atm) at 60°C were used for extractions. A solid phase trap packed with C18 was used to collect the extracted analytes. Trap temperature was set to +10°C when pure CO₂ was used as an extraction fluid, while trap temperature was set to 60°C when 15% ethanol-modified CO₂ was used. After completion of each extraction, the trap was rinsed with 10 mL or 50/50 mixture of ethanol/CH₂C₁₂. The extract volume was then adjusted to 25 mL using CH₂C₁₂.

Because there was no standard to determine extraction efficiency of kavalactones, all SFE extracts were compared with a liquid-solid extraction (LSE) of Kava root via a sonication method. The LSE was performed by sonicating 0.5 gram of Kava root with 10 mL of 50/50% MeOH/CH₂Cl₂ for 60 minutes at room temperature using a Fisher Scientific (Pittsburgh, PA) sonication bath. Next, the supernatant was filtered through a Gelman 0.45 µm nylon Acrodisc filter. The final volume was adjusted to 50 mL and analyzed via GC/MS. This extraction was assumed to yield 100% recovery of all kavalactones from the root.

A Hewlett-Packard G1205A Supercritical Fluid Chromatography (SFC) system with a variable UV detector equipped with a high pressure flow cell was used to obtain all SFC separations. Detection of lactones was monitored at 254 nm. The same instrument was used for semi-preparative scale separations but using the maximum flow rate (4 mL/min).

Table 5 lists the columns and the corresponding vendors that were used in this study. For semi-preparative scale separations, the column was 250 x 10 mm, dp = 5µm; whereas, analytical scale studies employed columns that were 250 x 4.6 mm, dp = 5µm. Seven kavalactones were identified in the supercritical extract using GC/MS (Hewlett Packard 5890 gas chromatography equipped with 5971A mass selective detector, and 7673 autosampler, Wilmington, DE). All GC separations were obtained on a 30 m x 0.25 mm i.d. x 0.25 µm dp DB-5 (J & W Scientific, Folsom, CA) fused silica capillary column. The column temperature was held at 50°C for 3 minutes, then programmed to 280°C at a rate of 10°C/min.

Table 5
Columns Used in This Study*

Column	Manufacture
Spherisorb NH2	Alltech
Altima Cyano	Alltech
Supelcosil LC-DIOL	Supleco
C4 Protein	Vatic
Diphenyl	Vatic

* 250 x 4.6 mm, 5F dp

HPLC grade methanol and ethanol were purchased from EM Science (Gibbstown, NJ). SFE/SFC grade CO₂ was used for both supercritical fluid extraction and supercritical fluid chromatography studies and was obtained from Air Products and Chemicals Co. (Allentown, PA).

5 Various conditions were used to obtain quantitative extraction of kavalactones from Kava root. Two pressures using both pure and 15% ethanol-modified CO₂ were studied to determine the extraction efficiency of kavalactones from Kava root. To determine the extraction efficiency of each lactone, an identical amount of Kava root was extracted via solid-liquid extraction (sonication) using 50/50% CH₂Cl₂/MeOH as
10 an extraction solvent. Results of the liquid-solid extraction were assumed to yield 100% recovery. Table 6 shows the relative extraction efficiency of each kavalactone extracted from Kava root under several SFE conditions. The results reveal that most of the kavalactones can be extracted with near critical or super critical gasses such as, for example, nitrogen, hydrogen or, preferably, butane, propane or freon. An efficiency of
15 greater than 90% was obtained using pure CO₂ at 350 atm and 60°C. Even higher extraction efficiency of kavalactones from Kava root can be obtained using 15% ethanol-modified CO₂. However, their extraction efficiency using pure CO₂ as an extraction fluid was less than 25%. This could be due to the larger sample size which was used in their extraction compared to results or the differences may be reflective of
20 the different trapping schemes used in the two studies.

Table 6
Percent Recoveries of Kavalactones from Kava Root Using SFE*

Compound	350 atm, 60°C	450 atm, 60°C	350 atm, 60°C	450 atm, 60°C
	100% CO ₂	100% CO ₂	85/15 CO ₂ /EtOH	85/15 CO ₂ /EtOH
25 7,8-Dihydrokavain	92.9 (7)	97.5 (6)	92.7 (2)	91.1 (5)
Kavain	93.6 (5)	100.0 (40)	102.9 (4)	107.0 (4)
5,6-Dihydrokavain	86.1 (8)	80.3 (5)	74.0 (8)	79.9 (7)
Dihydro-methysticin	93.2 (8)	88.4 (6)	95.3 (8)	104.1 (4)
Yanogonin	84.7 (11)	67.6 (12)	72.5 (9)	84.1 (9)
30 Methysticin	95.9 (7)	66.2 (10)	111.1 (7)	137.6 (12)
5,6,6,8-Tetra hydro-angonin	97.9 (5)	92.9 (8)	96.4 (4)	106.0 (6)

* = % recoveries are based upon comparison with 50/50 CH₂Cl₂/MeOH sonication extraction.

35 All extractions were performed at a flow rate of 2 mL/min for 60 minutes.

() = RSD for three replicate extractions.

Example 7 Supercritical Fluid Chromatography of Kavalactones- NH₂ Column

In the second part of this study, various columns with the same dimensions,
5 particle size (e.g. different stationary phases), and chromatography conditions were studied to optimize the SFC separation of kavalactones. An efficient analytical separation with supercritical fluid was felt to be advantageous in preparation for future scale-up work to isolate large quantities of each kavalactone.

Figure 25 shows the separation of kavalactone extract using an Alltech
10 Spherisorb NH₂ column. Separation was obtained isobarically at 125 atm and 60°C using a gradient of methanol-modified CO₂. The initial methanol concentration in CO₂ was 2% which was held constant for 3 minutes, and then MeOH was increased to 10% at a rate of 0.4%/minute. A separation of all kavalactones was obtained. However, the sixth peak eluted as a shoulder in front of the last major peak. To improve the separation of
15 the later eluting components both lower and higher temperatures were tested. Lowering the column temperature to 40°C caused co-elution of several peaks. Increasing the column temperature to 80°C not only provided baseline separation (Figure 26) for most of the kavalactones, but also higher resolution was obtained between peak 6 (t_R = 23.4 min). Increasing the pressure to 275 atm from 125 atm caused the kavalactones to elute
20 as only four peaks. It appeared that peaks 1 and 2, peaks 4 and 5, and peaks 6 and 7 co-eluted; while peak 3 eluted as one major peak.

Next, the modifier gradient was varied at 60°C in order to not only obtain a better separation but also to obtain the analysis in a shorter time. For this purpose, the initial modifier concentration was increased to 7%. After three minutes the modifier
25 concentration was increased to 10% at a rate of 0.2%/min. Figure 27 shows the resulting separation. As can be observed, increasing the initial modifier concentration not only provided a faster separation (analysis time of 12 minutes vs. 25 minutes), but also provided a separation with higher resolution of the last two peaks.

Example 8 Supercritical Fluid Chromatography of Kavalactones- C4 Protein Column

Most of the lactones co-eluted with a C4 protein column from Vatic using 125 atm, 70°C, 2 mL/min of liquid CO₂, and modifier programming (99/1% CO₂/MeOH hold for 4 minutes, and then increased to 97/3% CO₂/MeOH at rate of 0.1%/min). Increasing the oven temperature to 80, 90 and 100°C with a modifier program steadily improved the separation. Figure 28 shows a separation of the kavalactone extract using 100°C and 98.5/1.5% CO₂/MeOH as the initial mobile phase. Increasing the oven temperature provided baseline separation for most peaks. Increasing the density by increasing the column back pressure caused co-elution of several peaks. Decreasing the initial modifier concentration and the modifier gradient did not improve the separation at the higher pressure. Addition of 0.1% isopropyl amine (as a secondary modifier) to methanol prior to mixing in-line with CO₂ not only improved separation of the lactones but it also decreased the analysis time. Isopropyl amine provided more selectivity to obtain a better separation (Figure 29).

Example 9 SFC of Kavalactones- CN, DIOL and Diphenyl columns

Figure 30 shows the separation of the kavalactone extract on a Vatic diphenyl column. Unlike the other separations, the initial column back pressure was set to 125 atm for 3 minutes, which was increased to 195 atm at a rate of 5 atm/min. The initial mobile phase was 98/2% CO₂/MeOH which was increased to 93/7% CO₂/MeOH at a rate of 0.1%/min. The separation was obtained at 80°C at a flow rate of 2 mL/min. Only five peaks were observed. Peaks 2 and 3 co-eluted as well as peaks 6 and 7.

Figure 31 shows the SFC separation of kavalactones using an Altima CN column from Alltech at 125 atm, 60°C, and modifier programming starting with 98/2% CO₂/MeOH hold for 3 minutes and then increased to 90/10% CO₂/MeOH at a rate of 0.4%/min. As can be observed most of the analytes co-eluted. Increasing or decreasing either the temperature, pressure or modifier concentration failed to improve the separation. It is believed that this CN column did not have enough selectivity to resolve all the components.

Separation of the same extract on a Supelcosil DIOL column from Supleco was obtained. Chromatography conditions were 125 atm, 60°C, and a mobile phase composition of 98/2% CO₂/MeOH hold for 3 minutes and then increased to 90/10%

CO₂/MeOH at a rate of 0.4%/minute. Separation was similar to those obtained via the CN column. Again, temperature and modifier composition did not have a major effect on the separation.

Results from evaluation of these columns showed that NH₂ and protein C4 columns provided (almost) baseline separation of all kavalactones. However, it was believed that most of the peaks were resolved much better with the protein C4 column compared to the NH₂ column. Therefore, the protein C4 stationary phase was used to perform the semi-preparative separations.

Example 10 Semi-preparative Separation

Semi-preparative separation of kavalactones was first tried using a single protein C4 column, 250 x 10 mm, 5 µm dp. Parameters were changed to optimize the separation. The optimized separation employed 125 atm, 80°C, and a flow rate of 4 mL/min using a gradient of methanol-modified CO₂ (Figure 32). Results showed that a single protein C4 column did not have enough efficiency to separate all the kavalactones in the semi-preparative mode. Next, two semi-preparative protein C4 columns were connected in series to obtain the separation. Figure 33 shows the separation of the kavalactone extract (injection volume 5mg) using the previously stated conditions. As can be observed baseline separation of most of the kavalactones, in semi-preparative scale, were achieved using two columns connected in series.

Extraction of different kavalactones with efficiency greater than 90% was obtained using pure CO₂. However, higher extraction efficiency was obtained using 15% ethanol modified CO₂. Also, separation of different kavalactones from Kava root extract was performed using methanol modified supercritical CO₂. Results showed that separation of different kavalactones can be obtained using analytical scale amino and protein C4 columns. Semi-preparative separation of kavalactones was carried-out using two protein C4 columns connected in series. Baseline separation for most of the components were obtained.

Other embodiments and uses of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. All references cited herein, including all U.S. and foreign patents and patent

applications including U.S. provisional patent applications serial numbers 60/102,912, 60/122,526 and 60/136,409, and U.S. patent application serial numbers 09/408,922 and 09/518,191, are specifically and entirely incorporated by reference. It is intended that the specification and examples be considered exemplary only, with the true scope and spirit of the invention indicated by the following claims.

5

Claims

1. A method for extraction of at least one bioactive substance from at least one natural source, the method comprising the steps of:

contacting a non-chlorinated fluorocarbon solvent with the at least one natural source so that the solvent extracts a quantity of at least one bioactive substance from the at least one natural source;

removing the non-chlorinated fluorocarbon solvent to isolate the at least one bioactive substance.

2. The method of claim 1 wherein the at least one natural source is selected from the group consisting of Kava root, Byrsonima species, Aesculus californica, Crataegus mexicana, Simmondsia chinensis, Pfaffia species, Bursera species, Turnera species, Heimia salicifolia, Psidium species, Enterlobium species, Ptychopetalum olacoides, Liriosma ovata, and Chaunochiton kappleri.

3. The method of claim 2 wherein the non-chlorinated fluorocarbon solvent is 1,1,1,2-tetrafluoroethane

4. The method of claim 2 wherein the extraction is performed as a batch-wise, continuous cascading, or countercurrent process.

5. The method of claim 4 wherein the extraction is performed at at least one pressure of 0 to 10 bar.

6. The method of claim 4 wherein the extraction is performed at least one pressure of 3.5 to 5.6 bar.

7. The method of claim 2 wherein the extraction is performed with a mixture of the non-chlorinated fluorocarbon solvent and at least one other volatile substance.

8. The method of claim 7 wherein the at least one other volatile substance is selected from the group consisting of butane, propane, carbon dioxide, hexane, ethanol, methanol, and a combination thereof.

9. The method of claim 7 wherein the mixture of the non-chlorinated fluorocarbon solvent and the at least one other volatile substance is in a supercritical or near critical fluid state.

10. The method of claim 2 further comprising the step of processing the at least one natural source to make a powder, paste, maceration, or mixture prior to contacting the at least one natural source with the non-chlorinated fluorocarbon solvent.

11. The method of claim 2 wherein the at least one natural source is combined with at least one cosolvent so that the extraction is a liquid - liquid process.

12. The method of claim 11 wherein the at least one cosolvent is selected from the group consisting of alcohols, weak acids, ketones, chloro derivatives, hydrocarbons, fluorinated hydrocarbons, acetates, ethers, and a combination thereof.

13. The method of claim 2 wherein the at least one bioactive substance is a plurality of bioactive substances.

14. The method of claim 13 further comprising the step of separating the plurality of bioactive substances to obtain at least one isolated and purified bioactive substance.

15. The method of claim 14 wherein separating is achieved by high pressure liquid chromatography, packed column supercritical fluid chromatography, or radial flow chromatography.

16. A method for extraction of at least one bioactive substance from at least one natural source selected from the group consisting of Kava root, Byrsonima species, Aesculus californica, Crataegus mexicana, Simmondsia chinensis, Pfaffia species, Bursera species, Turnera species, Heimia salicifolia, Psidium species, Enterlobium species, Ptychopetalum olacoides, Liriosma ovata, and Chaunochiton kappleri, the method comprising the steps of contacting a non-chlorinated fluorocarbon solvent with the at least one natural source so that the solvent extracts a quantity of at least one bioactive substance from the at least one natural source wherein the extraction is a batch-wise, continuous cascading or countercurrent process; and removing the non-chlorinated fluorocarbon solvent to isolate the at least one bioactive substance.

17. A method for separating analytes contained in an extract, the method comprising the steps of:

running at least one volatile substance through a packed column, the at least one volatile substance being in a near-critical or supercritical fluid state;

passing the extract through the packed column; and

collecting the analytes which have been separated.

wherein the analytes are at least one bioactive substance and the extract is from a natural source.

18. The method of claim 17 wherein the at least one volatile substance is selected
5 from the group consisting of ethanol, methanol, butane, propane, dichloromethane, tetrafluoroethane, isopropyl amine, and a combination thereof.

19. The method of claim 17 wherein the packed column is selected from the group consisting of a C4 protein column, a NH₂ column, a CN column, a DIOL column, and a diphenyl column.

10 20. The method of claim 17 wherein the natural source is selected from the group consisting of Kava root, Byrsonima species, Aesculus californica, Crataegus mexicana, Simmondsia chinensis, Pfaffia species, Bursera species, Turnera species, Heimia salicifolia, Psidium species, Enterlobium species, Ptychopetalum olacoides, Liriosma ovata, and Chaunochiton kappleri.

15 21. A continuous cascading extraction method for extracting a plurality bioactive substances from at least one natural source, the method comprising the steps of:

placing a quantity of the at least one natural source into a plurality of extraction vessels;

20 passing a volatile substance through the plurality of extraction vessels in a continuous manner until a desired concentration of the plurality of bioactive substances in the volatile substance is reached;

removing the volatile substance to obtain a quantity of the plurality of bioactive substances.

22. The method of claim 21 wherein the at least one natural source is selected from
25 the group consisting of Kava root, Byrsonima species, Aesculus californica, Crataegus mexicana, Simmondsia chinensis, Pfaffia species, Bursera species, Turnera species, Heimia salicifolia, Psidium species, Enterlobium species, Ptychopetalum olacoides, Liriosma ovata, and Chaunochiton kappleri.

23. The method of claim 21 wherein the volatile substance is a non-chlorinated
30 fluorocarbon solvent.

24. The method of claim 23 wherein the non-chlorinated fluorocarbon solvent is 1,1,1,2-tetrafluoroethane.

25. The method of claim 21 wherein the extraction is performed at a pressure of 0 to 10 bar.

5 26. The method of claim 21 wherein the extraction is performed with a mixture of the non-chlorinated fluorocarbon solvent and at least one other volatile substance.

27. The method of claim 26 wherein the at least one other volatile substance is selected from the group consisting of butane, propane, carbon dioxide, hexane, ethanol, methanol, nitrogen, chloroform and combinations thereof.

10 28. The method of claim 26 wherein the mixture of the non-chlorinated fluorocarbon solvent and the at least one other volatile substance is in a supercritical or near critical fluid state.

29. The method of claim 21 further comprising the step of processing the at least one natural source to make a powder, paste, maceration, or mixture prior to placing the at
15 least one natural source into the extraction vessel.

30. The method of claim 21 wherein the at least one natural source is combined with at least one cosolvent so that the extraction is a liquid - liquid process.

31. The method of claim 30 wherein the at least one cosolvent is selected from the group consisting of alcohols, weak acids, ketones, chloro derivatives, hydrocarbons, fluorinated hydrocarbons, acetates, ethers and combinations thereof.
20

32. The method of claim 21 further comprising the step of separating the plurality of bioactive substances to obtain at least one isolated and purified bioactive substance.

33. The method of claim 32 wherein separating is achieved by radial flow chromatography, high pressure liquid chromatography or packed column supercritical
25 fluid chromatography.

34. An ingestible formula for treating neurological and vascular disorders comprising a therapeutic concentration of at least one bioactive substance extracted from a Byrsonima species by a continuous cascading extraction with a non-chlorinated fluorocarbon solvent.

35. The formula of claim 34 further comprising a therapeutic concentration of at least one bioactive substance from at least one other natural source.

36. The formula of claim 35 wherein the at least one other natural source is selected from the list consisting of *Psidium* species, *Enterlobium* species, and a combination thereof.

37. The formula of claim 36 wherein the *Byrsonima* species is *Byrsonima crassifolia*, the *Psidium* species is *Psidium guajava*, and the *Enterlobium* species is *Enterlobium cyclocarpum*.

38. The formula of claim 34 which is in the form of a tablet, capsule, pastille, or elixir.

39. An ingestible formula comprising a therapeutic quantity of at least one bioactive substance extracted from a *Byrsonima* species and a therapeutic quantity of at least one bioactive substance extracted from a *Psidium* species, an *Enterlobium* species, or a combination thereof, wherein the at least one bioactive substance extracted from a *Byrsonima* species and the at least one bioactive substance extracted from a *Psidium* species, an *Enterlobium* species, or a combination thereof are extracted with an organic solvent, water, an organic solvent/water mixture, a supercritical fluid extraction, a dense gas extraction or combinations thereof.

40. The formula of claim 39 wherein the organic solvent is methanol, ethanol, ethyl acetate or combinations thereof.

41. The formula of claim 39 wherein the *Byrsonima* species is *Byrsonima crassifolia*, the *Psidium* species is *Psidium guajava*, and the *Enterlobium* species is *Enterlobium cyclocarpum*.

42. The formula of claim 41 wherein the therapeutic first quantity at least one bioactive substance extracted from a *Byrsonima* species is selected from the group consisting of β -sitosterol, betulin, proline, pipercolic acid, quercetin, catechin, and a combination thereof.

43. An ingestible formula for use as a cardiovascular tonic comprising a therapeutic concentration of a plurality of bioactive substances extracted from *Aesculus* species and

Crataegus species by a continuous cascading extraction with a non-chlorinated fluorocarbon solvent.

44. The formula of claim 43 further comprising a therapeutic concentration of at least one bioactive substance from at least one other natural source.

5 45. The formula of claim 44 wherein the at least one other natural source is a Bursera species.

46. The formula of claim 45 wherein the Aesculus species is Aesculus californica, the Crataegus species is Crataegus mexicana, and the Bursera species is Bursera microphylla.

10 47. The formula of claim 43 which is in the form of a tablet, capsule, pastille, or elixir.

48. An ingestible formula comprising a therapeutic quantity of at least one bioactive substance extracted from an Aesculus species and a therapeutic quantity of at least one bioactive substance extracted from a Crataegus species, wherein the at least one
15 bioactive substance extracted from an Aesculus species and the at least one bioactive substance extracted from a Crataegus species are extracted with an organic solvent, water, an organic solvent/water mixture, a near-critical or supercritical fluid extraction, a dense gas extraction, or a combination thereof.

49. The formula of claim 48 wherein the Aesculus species is Aesculus californica,
20 and the Crataegus species is Crataegus mexicana.

50. The formula of claim 48 wherein the organic solvent is methanol, ethanol, ethyl acetate, or a combination thereof.

51. The formula of claim 48 wherein the therapeutic quantity of at least one bioactive substance extracted from an Aesculus species is selected from the group consisting of
25 β -methyl alanine, phenylalanine, isohomoleucine, isohomo-6-hydroxyleucine, mino-4-methyl-hex-trans-4-enoic acid, gamma-glutamyl-2-A-hex-4-enoic acid, arbutin, hydroquinone, epicatechin, coumarin eleutheroside B-1, quebrachitol, and a combination thereof.

52. An extract of Jojoba for satiating hunger and reducing weight in humans, the
30 extract comprising simmondsin, wherein the simmondsin is extracted from the Jojoba

with an organic solvent, water, an organic solvent/water mixture, a near-critical or supercritical fluid extraction, a dense gas extraction, or a non-chlorinated fluorocarbon solvent.

53. The extract of claim 52 wherein the simmondsin is extracted from a defatted meal of Jojoba.

54. The extract of claim 52 wherein the non-chlorinated fluorocarbon solvent is 1,1,1,2-tetrafluoroethane.

55. The extract of claim 52 wherein the simmondsin is extracted from the Jojoba with a continuous cascading extraction method.

56. The extract of claim 52 which is in the form of a tablet, capsule, pastille or elixir.

57. A method for satiating hunger in a human being to aid in weight reduction, the method comprising administering an extract of Jojoba comprising simmondsin to the human being.

58. The method of claim 57 wherein the extract is in the form of a tablet, capsule, pastille or elixir.

59. The method of claim 57 wherein the extract is obtained from a defatted meal of Jojoba by extraction with a non-chlorinated fluorocarbon solvent.

60. An ingestible formula for use as a health tonic and to support sexual function comprising a plurality of bioactive substances extracted from *Turnera* species and *Pfaffia* species by a continuous cascading extraction with a non-chlorinated fluorocarbon solvent.

61. The formula of claim 60 wherein the sexual function supported is male, female or both.

62. The formula of claim 60 further comprising a therapeutic concentration of at least one bioactive substance from at least one other natural source.

63. The formula of claim 62 wherein the at least one other natural source is selected from the group consisting of *Ptychopetalum olacoides*, *liriosma ovata*, *Chaunochiton kappleri*, *muira pauma*, and a combination thereof.

64. The formula of claim 60 wherein the *Turnera* species is *Turnera diffusa*, and the *Pfaffia* species is *Pfaffia paniculata*.

65. The formula of claim 60 which is in the form of a tablet, capsule, pastille, or elixir.

66. An ingestible formula comprising a therapeutic quantity of at least one bioactive substance extracted from a *Turnera* species and a therapeutic quantity of at least one bioactive substance extracted from a *Pfaffia* species, wherein the at least one bioactive substance extracted from a *Turnera* species and the at least one bioactive substance extracted from a *Pfaffia* species, are extracted with an organic solvent, water, an organic solvent/water mixture, a supercritical fluid extraction, a dense gas extraction, or a combination thereof.

67. The formula of claim 66 wherein the *Turnera* species is *Turnera diffusa*, and the *Pfaffia* species is *Pfaffia paniculata*.

68. The formula of claim 66 wherein the organic solvent is methanol, ethanol, ethyl acetate, or a combination thereof.

69. The formula of claim 66 wherein the therapeutic first quantity at least one bioactive substance extracted from a *Turnera* species is selected from the group consisting of β -sitosterol, arbutin, caffeine, gonzalitosin, hexacosan-1-ol, tetraphyllin B, N-triacontane, tricosan-2-one, paracymene, α -pinene, β -pinene and combinations thereof.

70. The formula of claim 66 wherein the therapeutic first quantity at least one bioactive substance extracted from a *Pfaffia* species is selected from the group consisting of allantoin, daucosterol, β -ecdysone, pfaffic acid, pfaffosides A, B, C, D, E, and F, polypodine B, β -sitosterol, stigmasterol, stigmasterol-3-O- β -D-glucoside and combinations thereof.

71. An ingestible formula for use as a non-steroidal anti-inflammatory comprising a plurality of bioactive substances extracted from *Heimia* species by an organic solvent, water, an organic solvent/water mixture, a near-critical or supercritical fluid extraction, a dense gas extraction, or a continuous cascading extraction with a non-chlorinated fluorocarbon solvent.

72. The formula of claim 71 further comprising a therapeutic concentration of at least one bioactive substance from at least one other natural source.

73. The formula of claim 71 wherein the *Heimia* species is *Heimia salicifolia*.

74. The formula of claim 71 which is in the form of a tablet, capsule, pastille, or elixir.

75. An ingestible formula comprising a therapeutic quantity of at least one bioactive
5 substance extracted from a *Heimia* species, wherein the at least one bioactive substance
extracted from a *Heimia* species, are extracted with an organic solvent, water, an organic
solvent/water mixture, a near-critical fluid extraction, a supercritical fluid extraction, a
dense gas extraction, or a combination thereof.

76. The formula of claim 75 wherein the *Heimia* species is *Heimia salicifolia*.

10 77. The formula of claim 75 wherein the organic solvent is methanol, ethanol, ethyl
acetate or combinations thereof.

78. The formula of claim 75 wherein the therapeutic first quantity at least one
bioactive substance extracted from a *Heimia* species is selected from the group
consisting of cryogenine, nesodine, vertine, lytrine, lyfoline, demethoxyabresoline,
15 epidemethoxylabresoline, demethyllasubine-I, demethyllasubine-II and combinations
thereof.

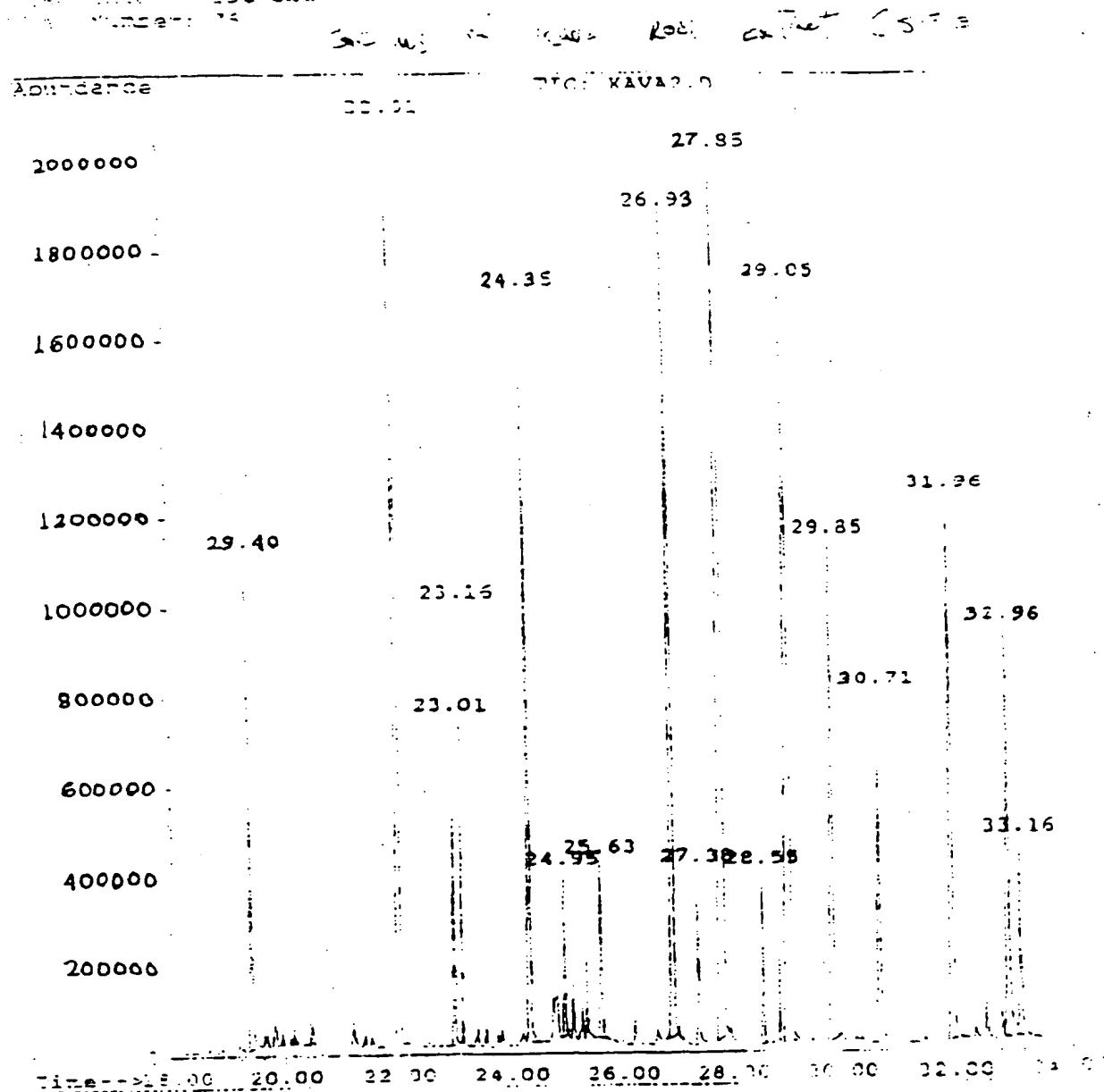
1/33

Figure 1

```

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Operator      : mehdi
Acquired      : 28 May 98   1:23 pm using AcqMethod KAVAMAN
Instrument     : 5972 - GC
Sample Name    : Kava Extract pure CO2 350 atm 160C 60min, 2m
               : 1st ext
               :
Acquisition    :

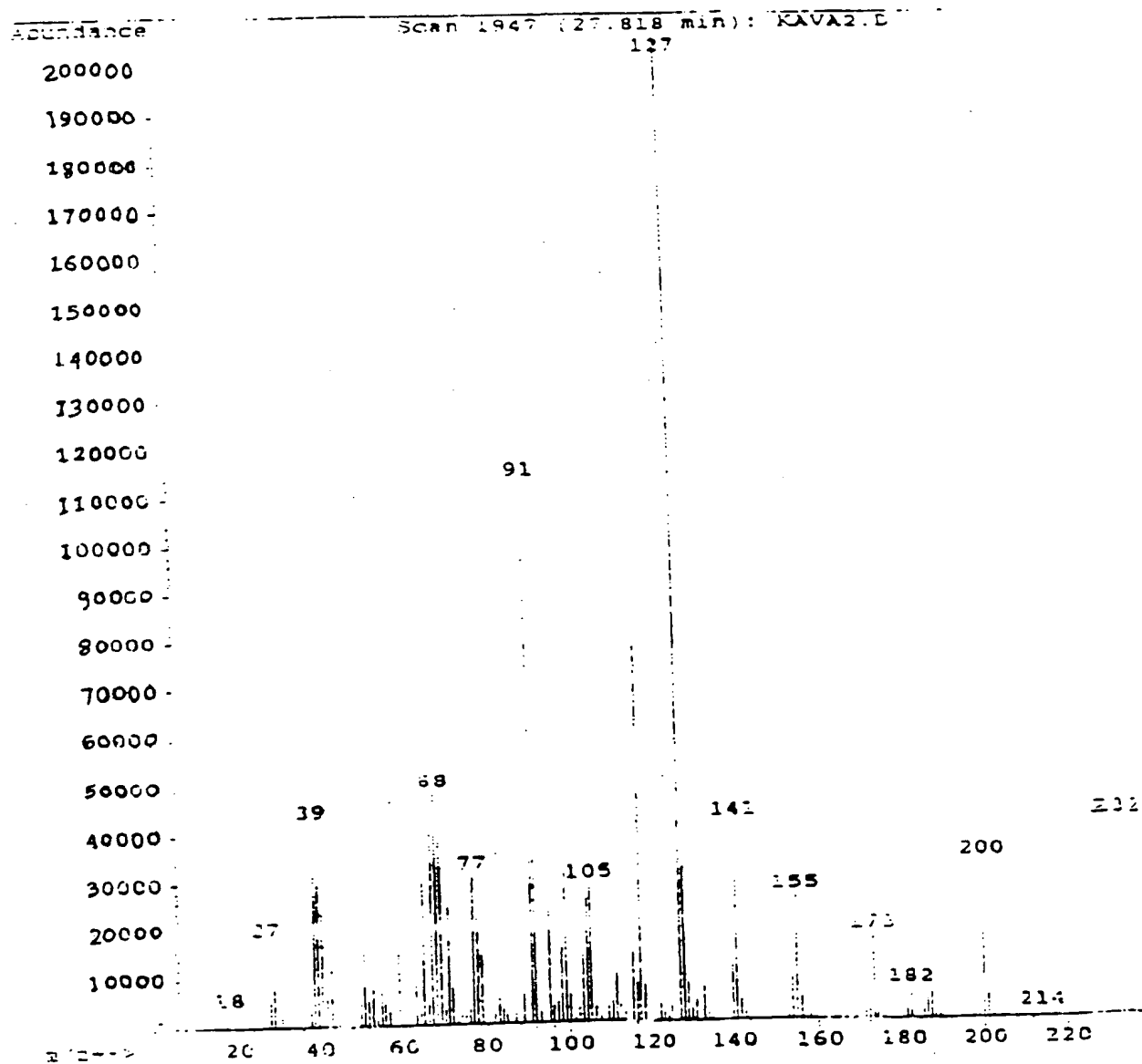
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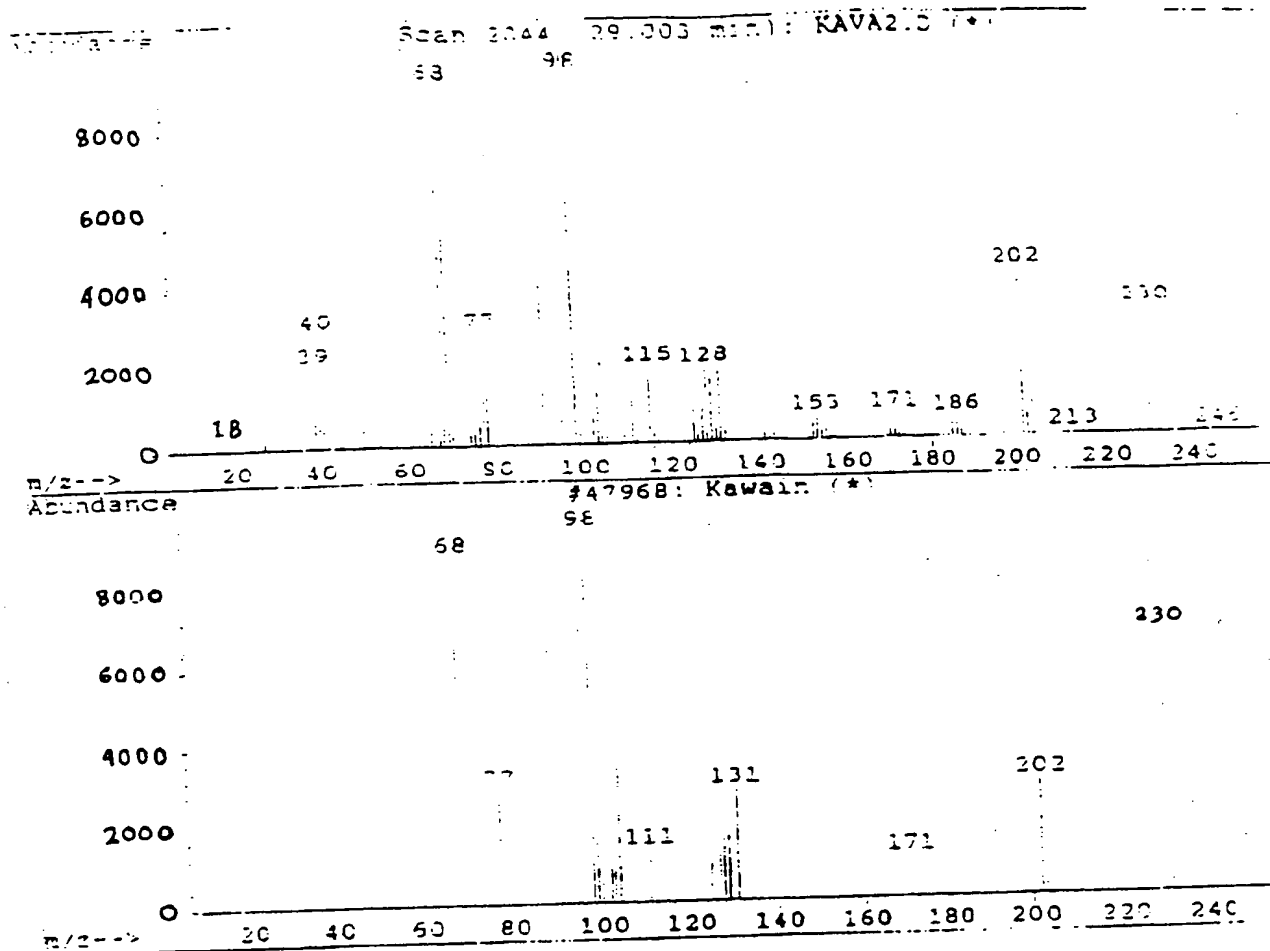
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Instrument: 5972 - GC
Sample Name: Kava Extract pure CO2 150 atm, 60C, 60min, 2m
Misc Info: 1st ext
Vial Number: 76



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 #1
 KAWAIA

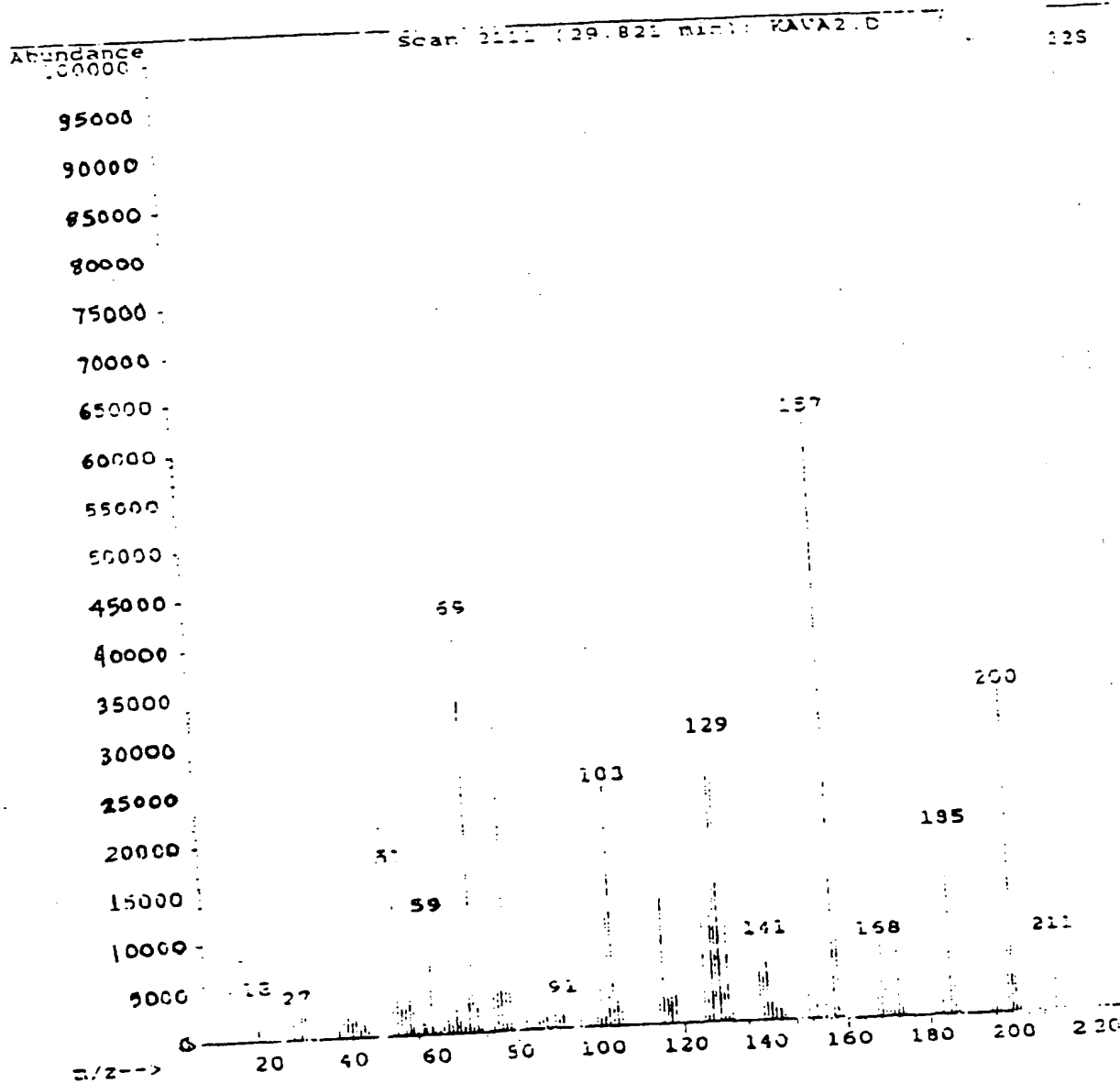
Figure 3



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Figure 4

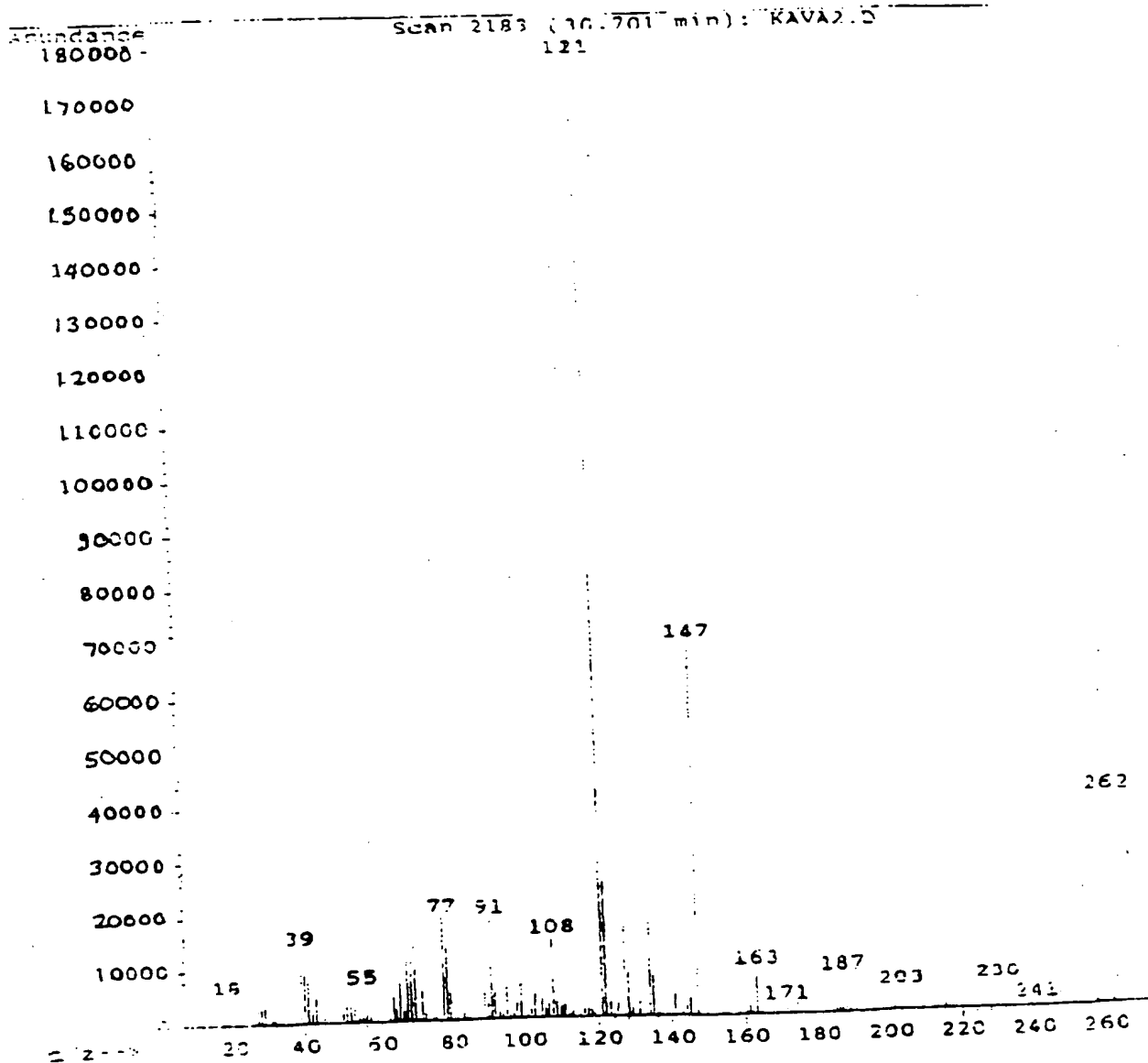
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Sample Name: Kava Extract pure 002 050 atm 500 50min 2m
Scan Info: 1st ext
Scan Number: 5972



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Figure 5

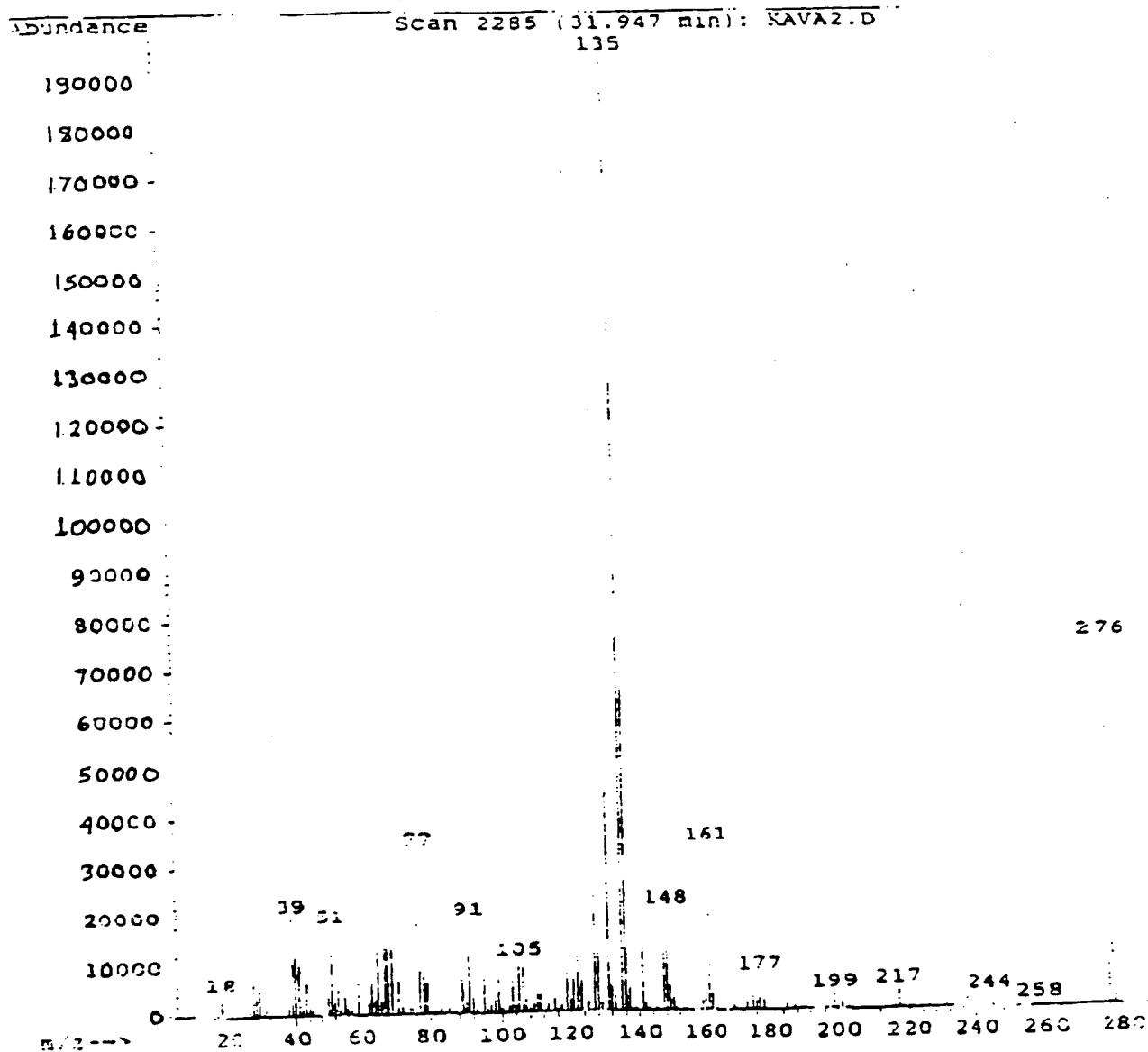
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Sample Name: Kava Extract pure CO2 350 atm 600. 60min. 2m
Vial Info: 1st ext
Vial Number: 76



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Figure 6

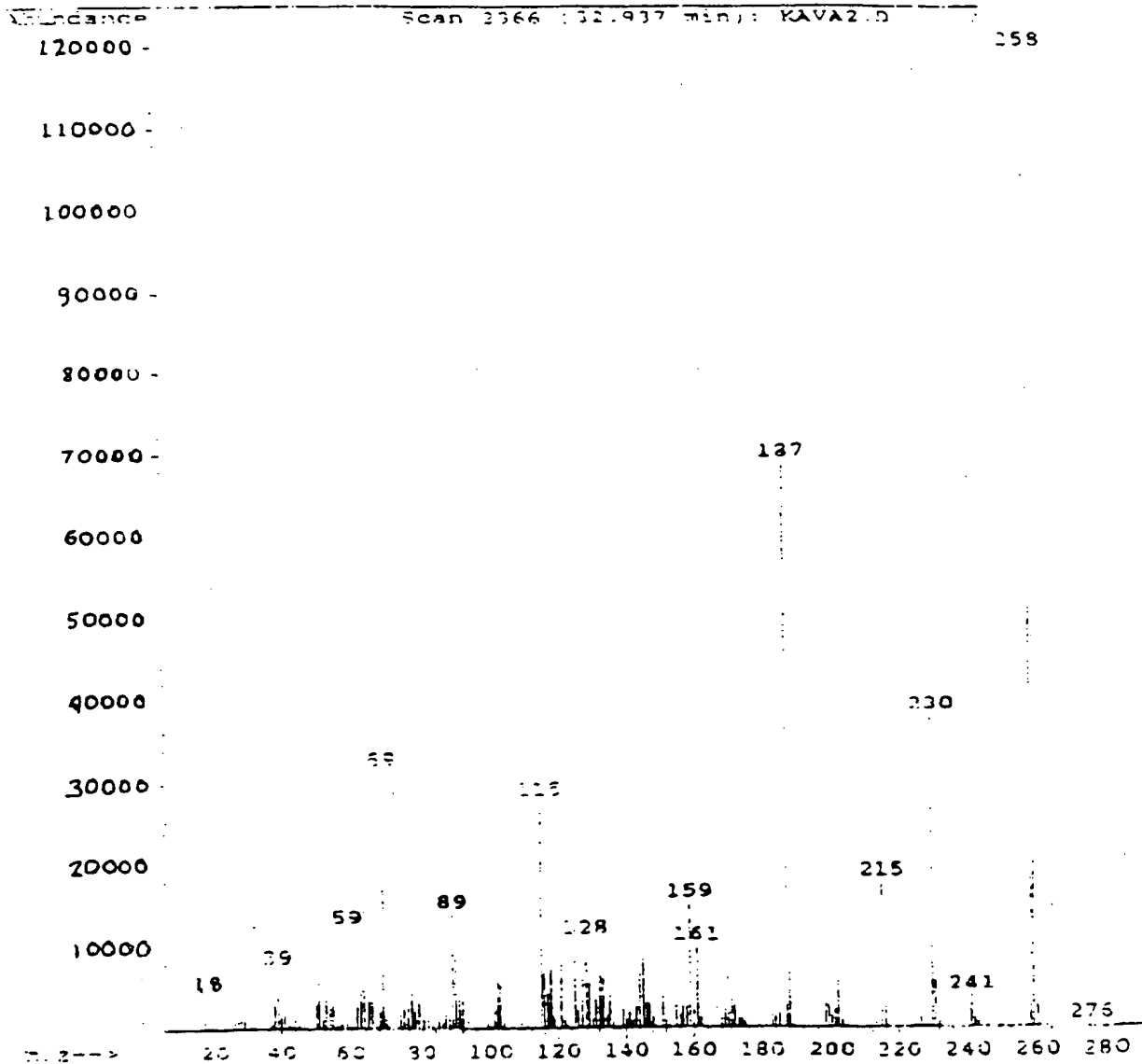
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Operator: mehdi
Acquire: 28 May 98 12:21 pm using AcqMethod KAVAMAK
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Acq Info: 1st ext
File Number: 26



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Figure 7

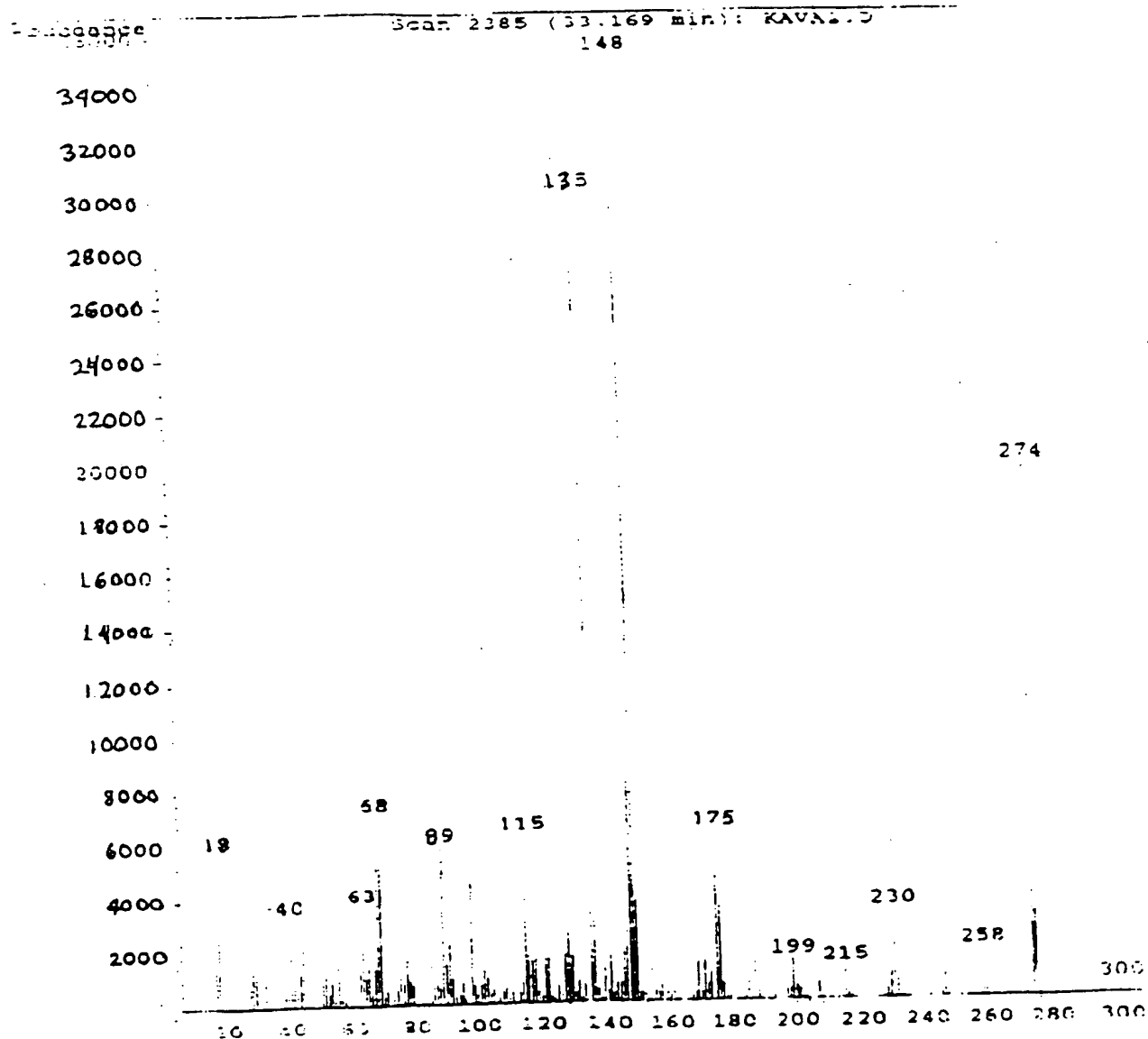
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Operator : mehdi
Acquired : 28 May 98 1:23 pm using AcqMethod KAVAMAK
Instrument : 5972 - GC
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Scan Info 1st ext
Scan Number: 78



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Figure 8

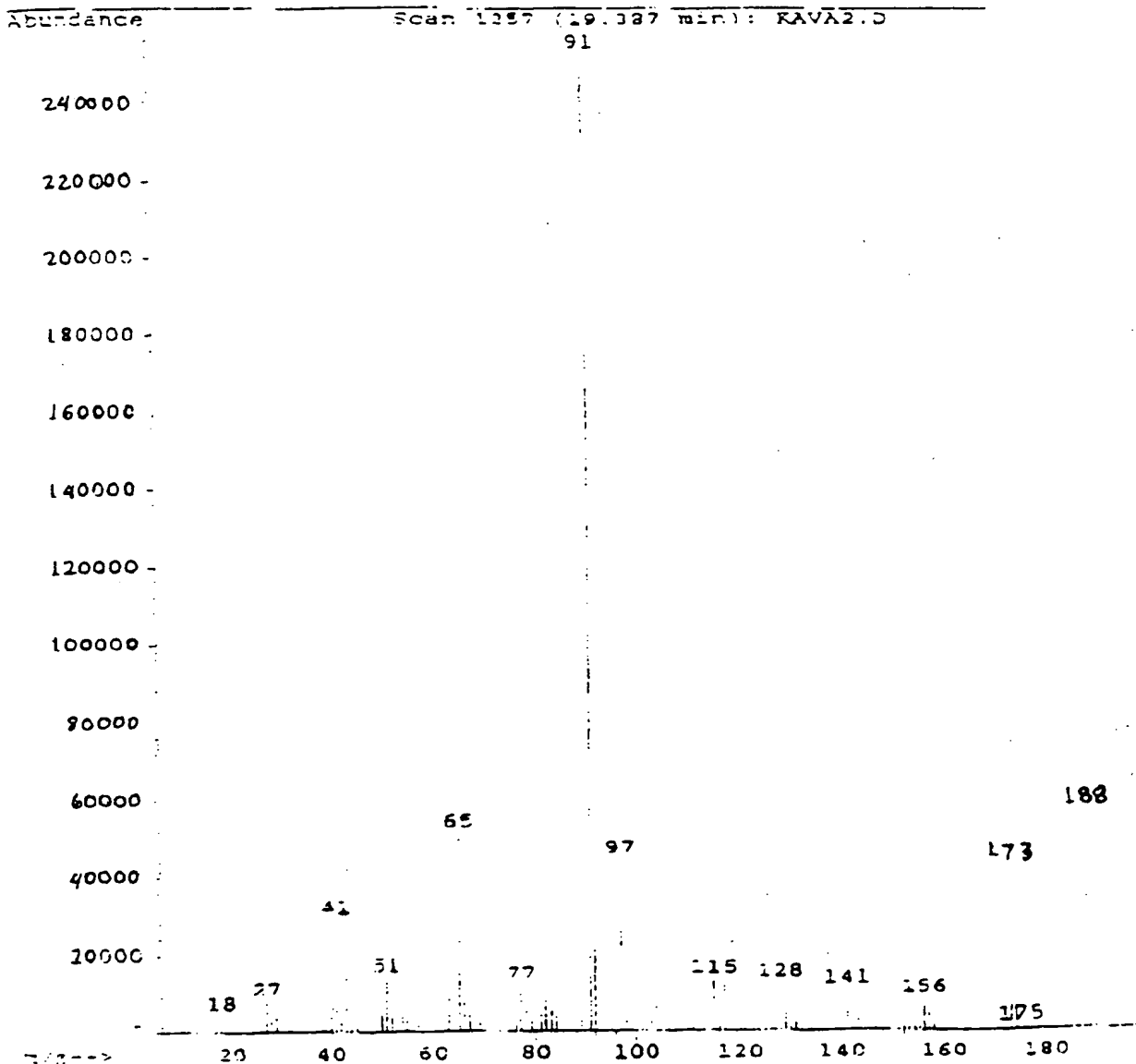
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Instrument: 5972 - GC
Sample Name: Kava Extract pure CO2 150 atm, 60C, 60min, 2m
Misc Info: 1st ext
Vial Number: 78



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Figure 9

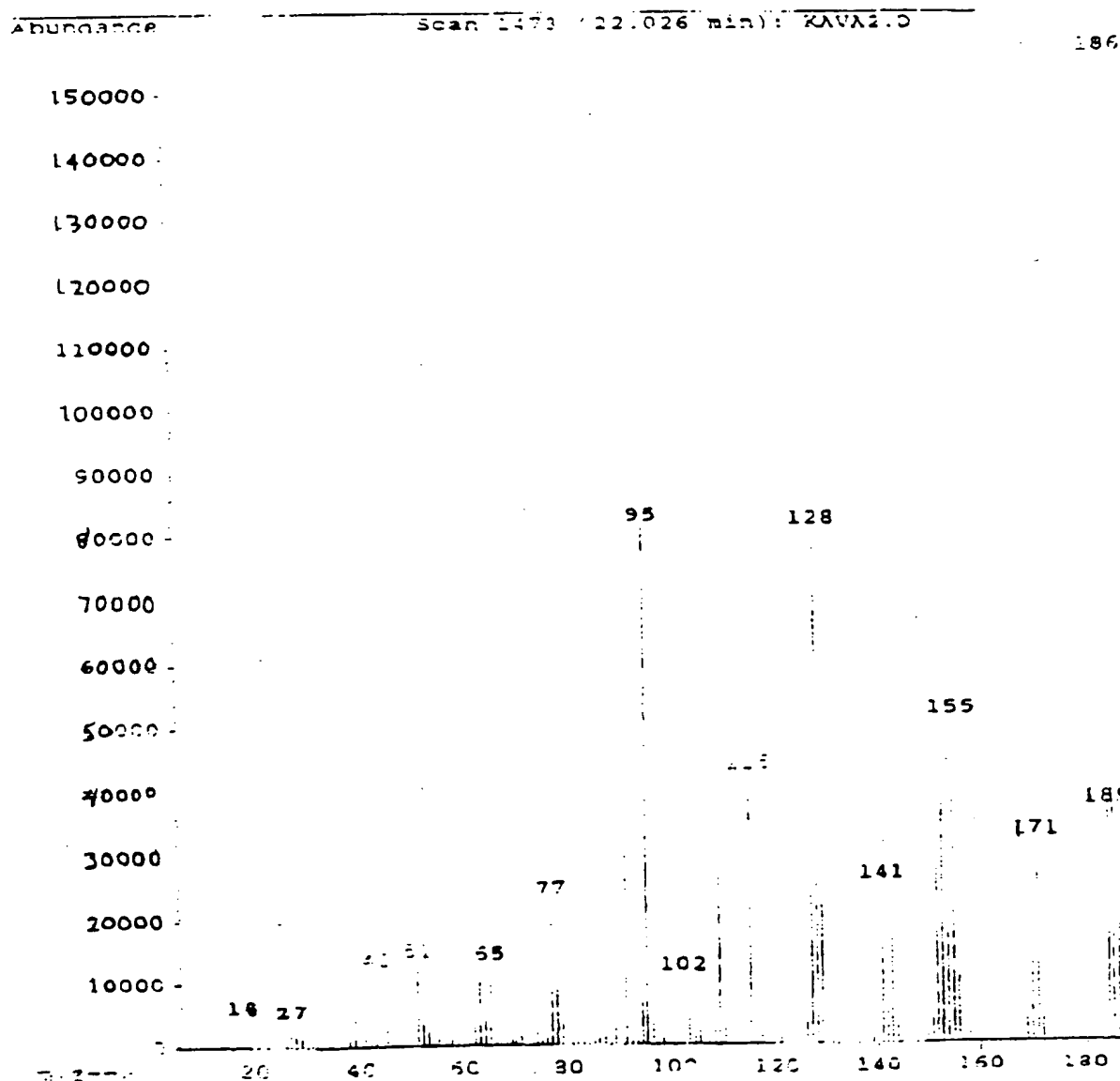
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Acquired: 12 May 98 11:23 am using AcqMethod KAVAMAK
Instrument: 5972 - GC
Sample Name: Kava Extract pure CO2 350 atm, 600, 60min, 2m
Misc Info: 1st ext.
Scan Number: 16



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Figure 10

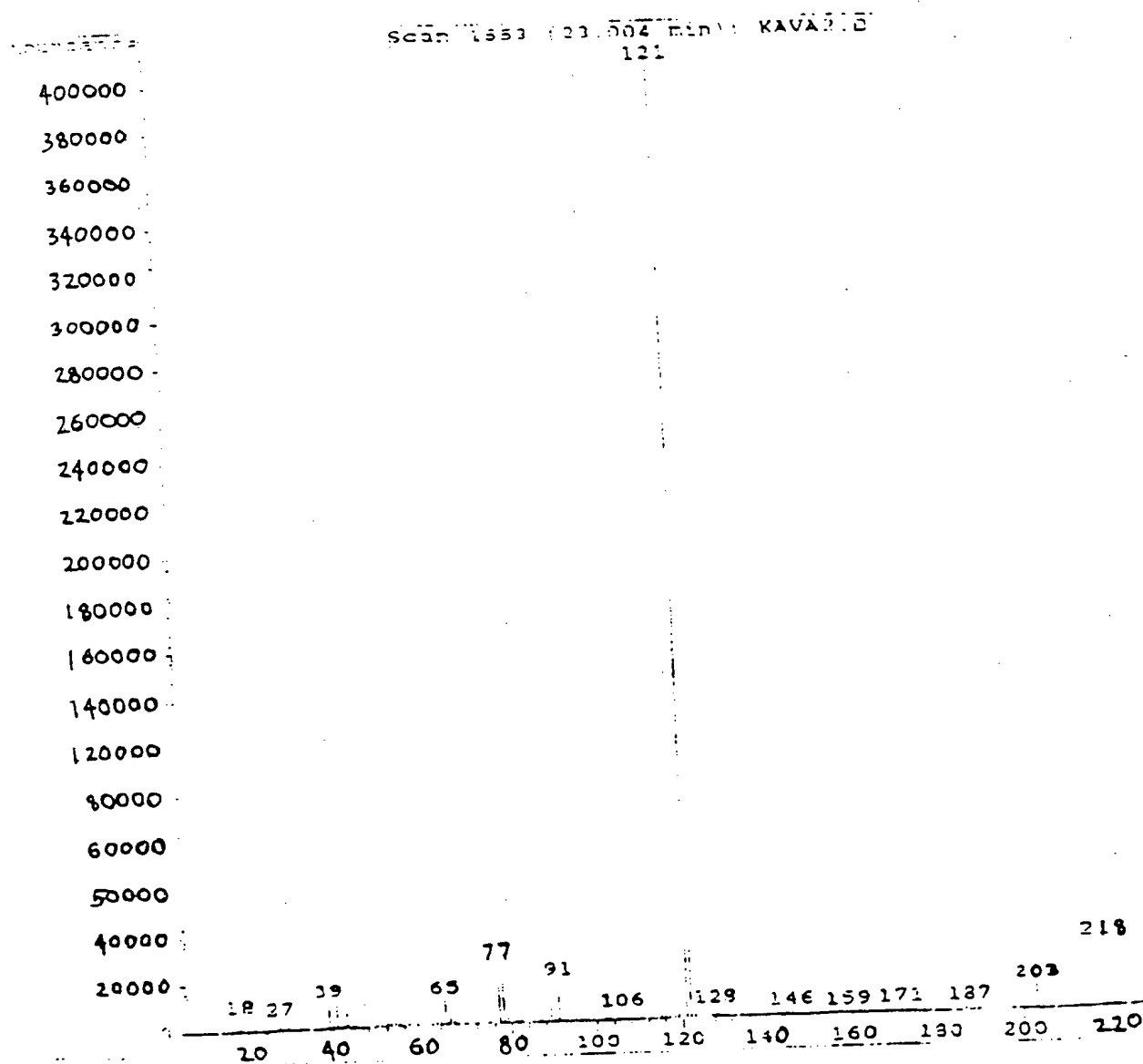
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File Info: 1st ext
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Figure 11

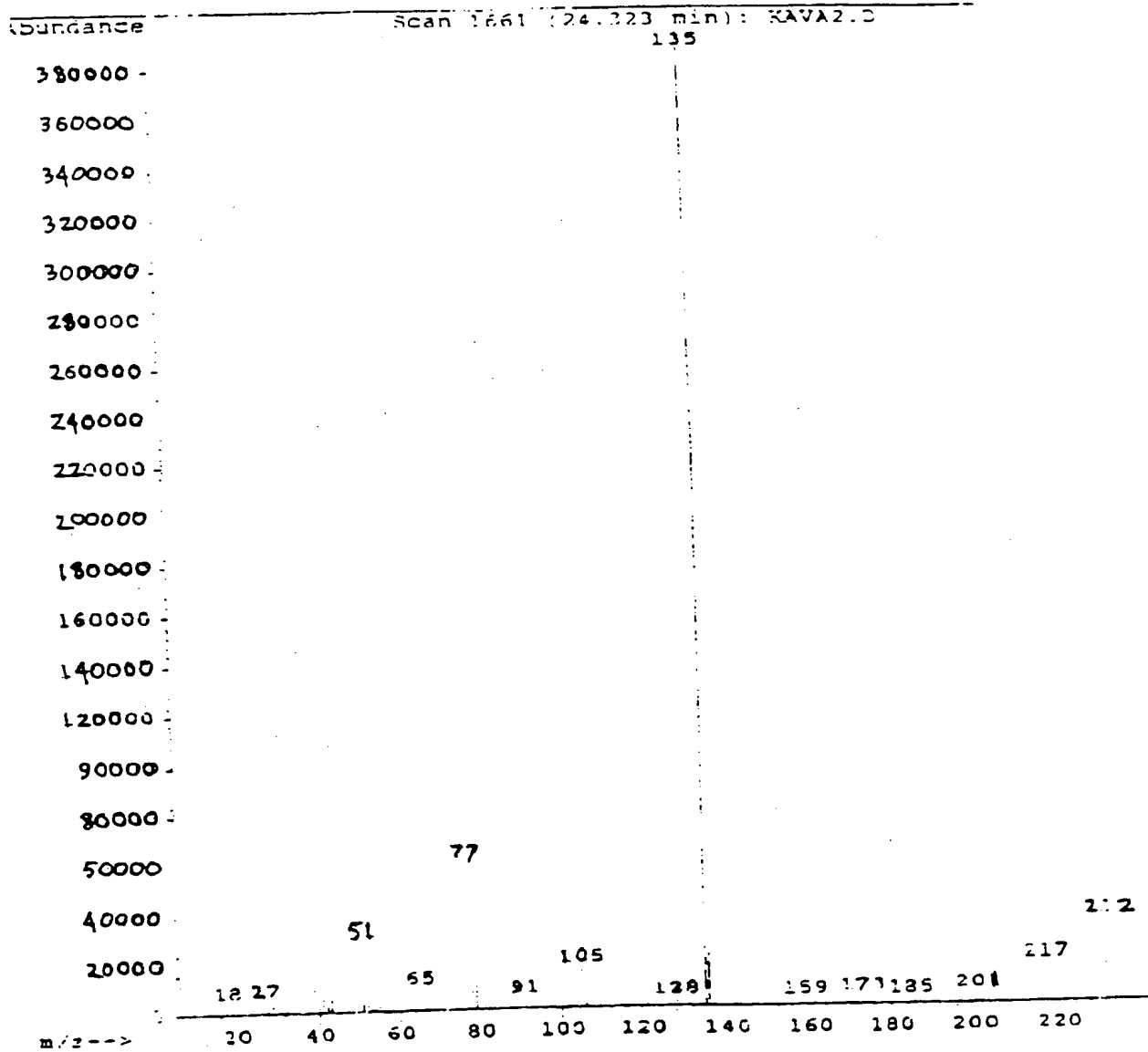
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Operator: Mehdi
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Sample Name: Kava Extract pure CO2 350 atm 600 60min.0m
File Name: 150 ext
File Number: 75



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Figure 12

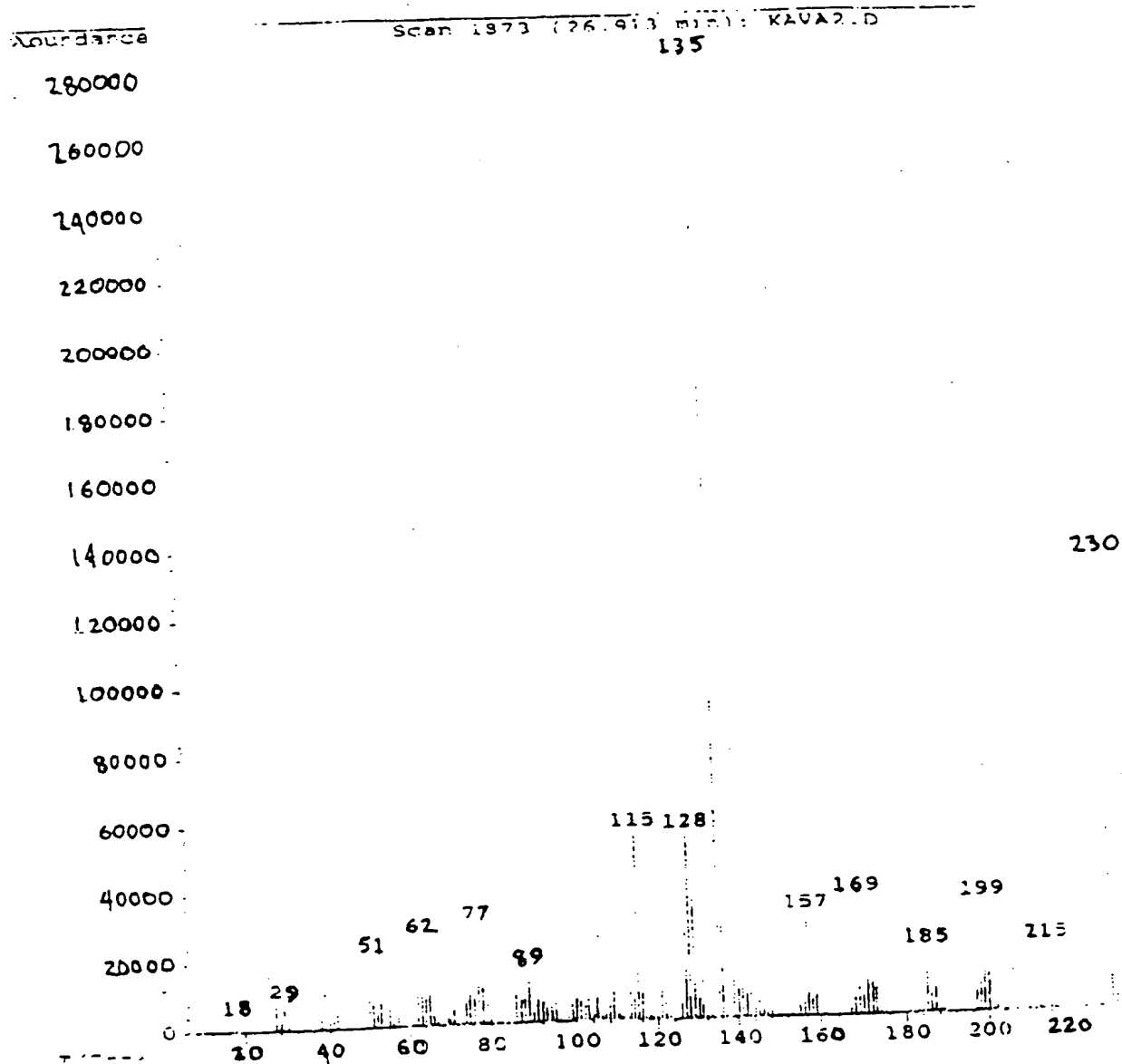
\\HPCHEM\1\DATA\KAVA2.D
Operator: mehdi
Acquired: 25 May 98 1:23 pm using AcqMethod KAVAMAK
Instrument: 5972 - GC
Sample Name: Kava Extract pure 102 150 atm 150C 60min.2m
File Info: 1st ext
Data Number: 76



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Figure B

HPCHEM1\DATA\KAVA2.D
Method: mendi
Date: 23 May 98
AcqMethod: KAVAMAX
Sample Name: Kava Extract pure
Scan 1973 (26.913 min): KAVA2.D
135



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Figure 14

DATA: HP0CHEM\1\DATA\KAWAMAK\KAWA1_2.D
Operator: MF
Acquired: 29 May 98 3:25 pm using AcqMethod KAWAMAK
Sample: 5972 - GC
Injection: sonication extraction of 0.5 g sample Me2012
Injection Volume: 1.0
Injection Number: 15

GC/MS of Kava Root extract (sonication)

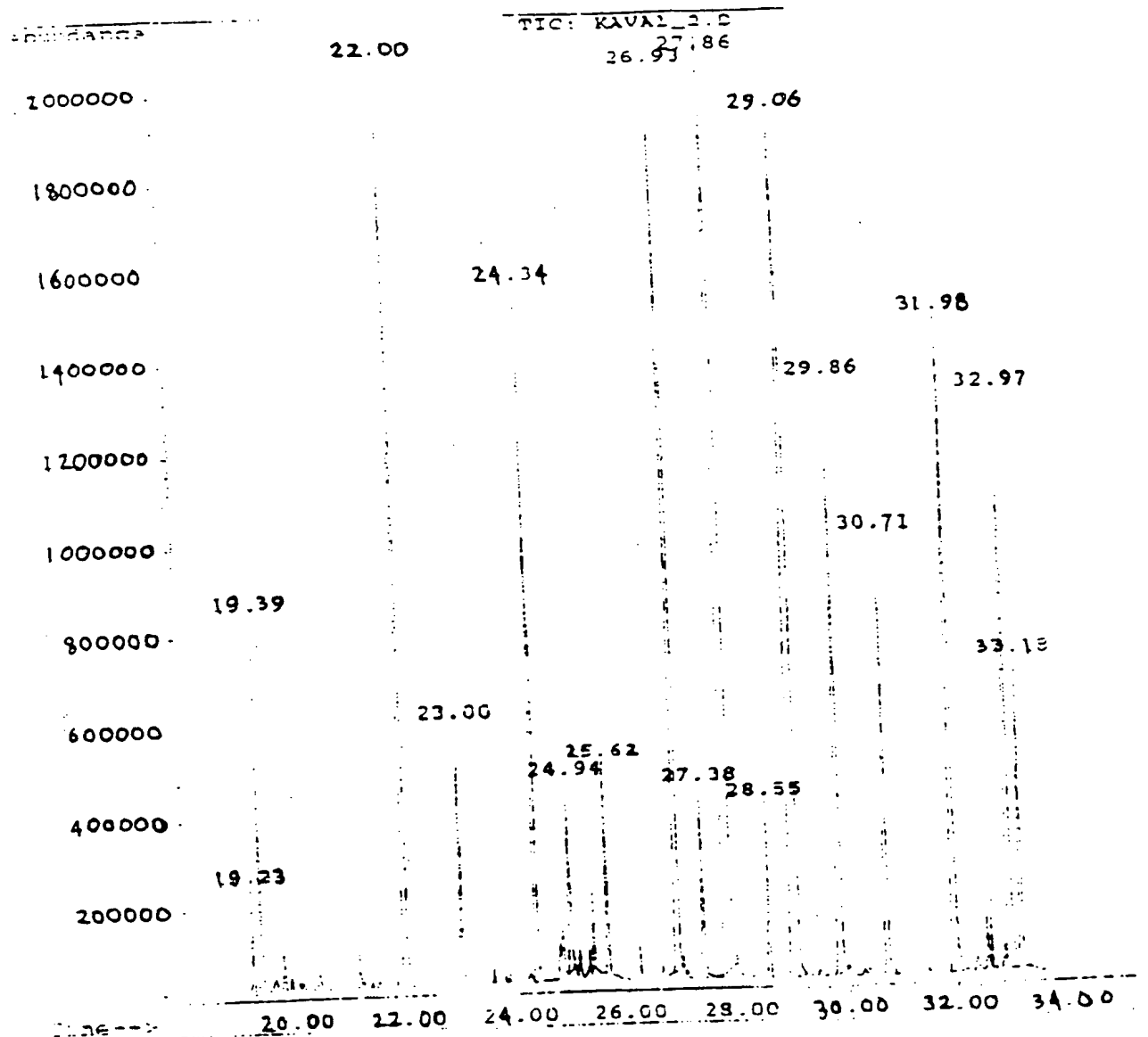


Figure 15

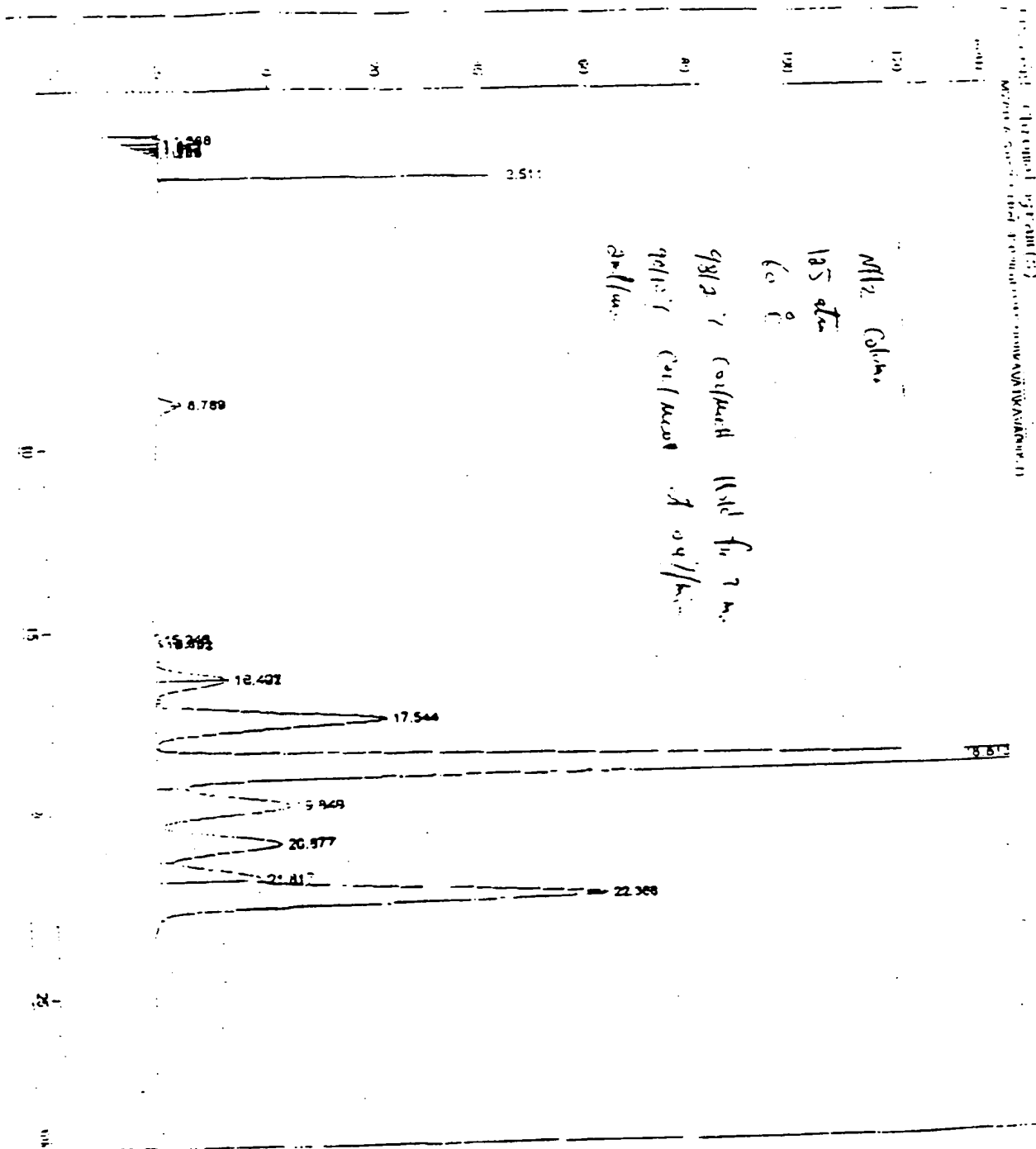


Figure 1b

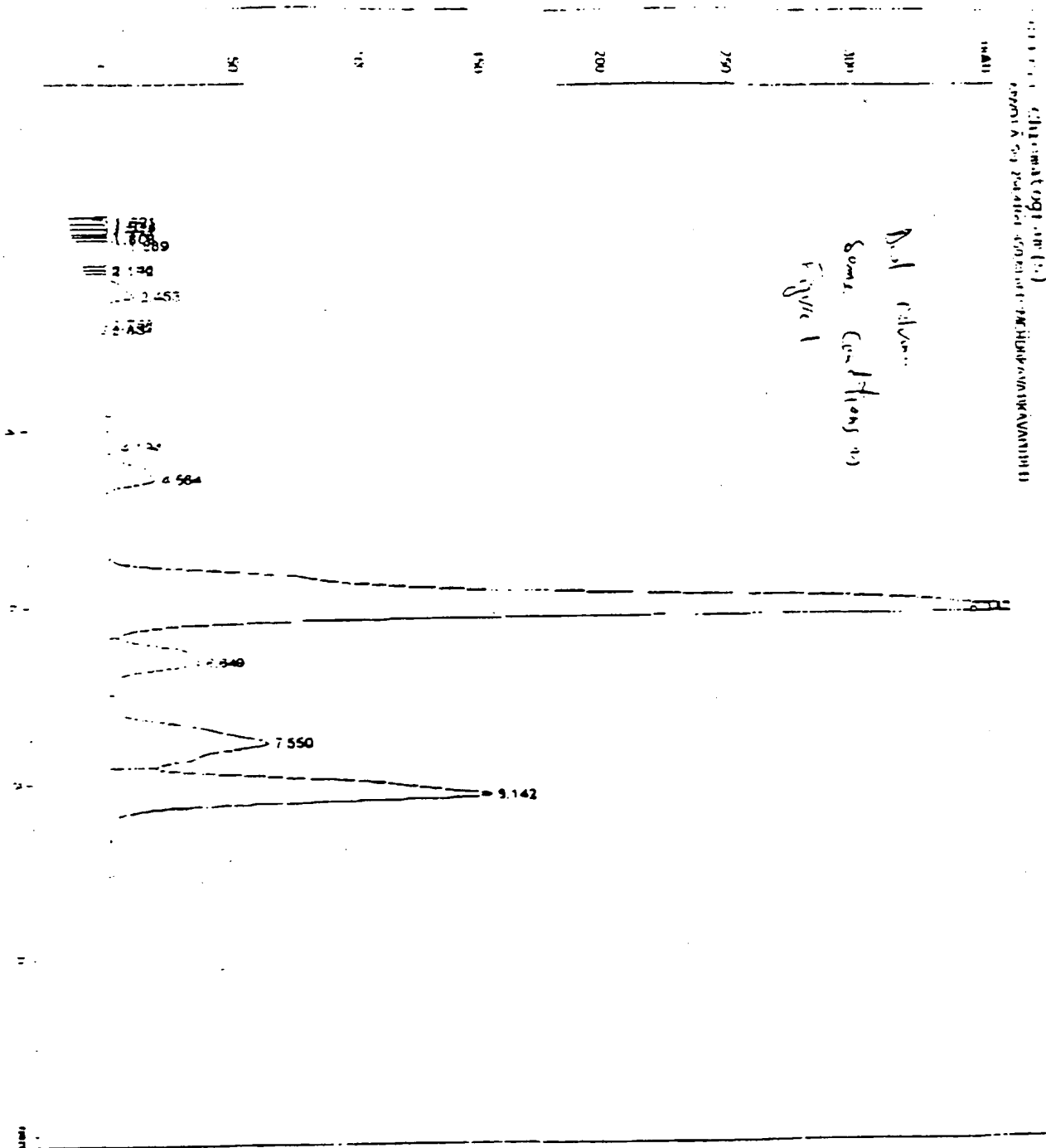


Figure 17

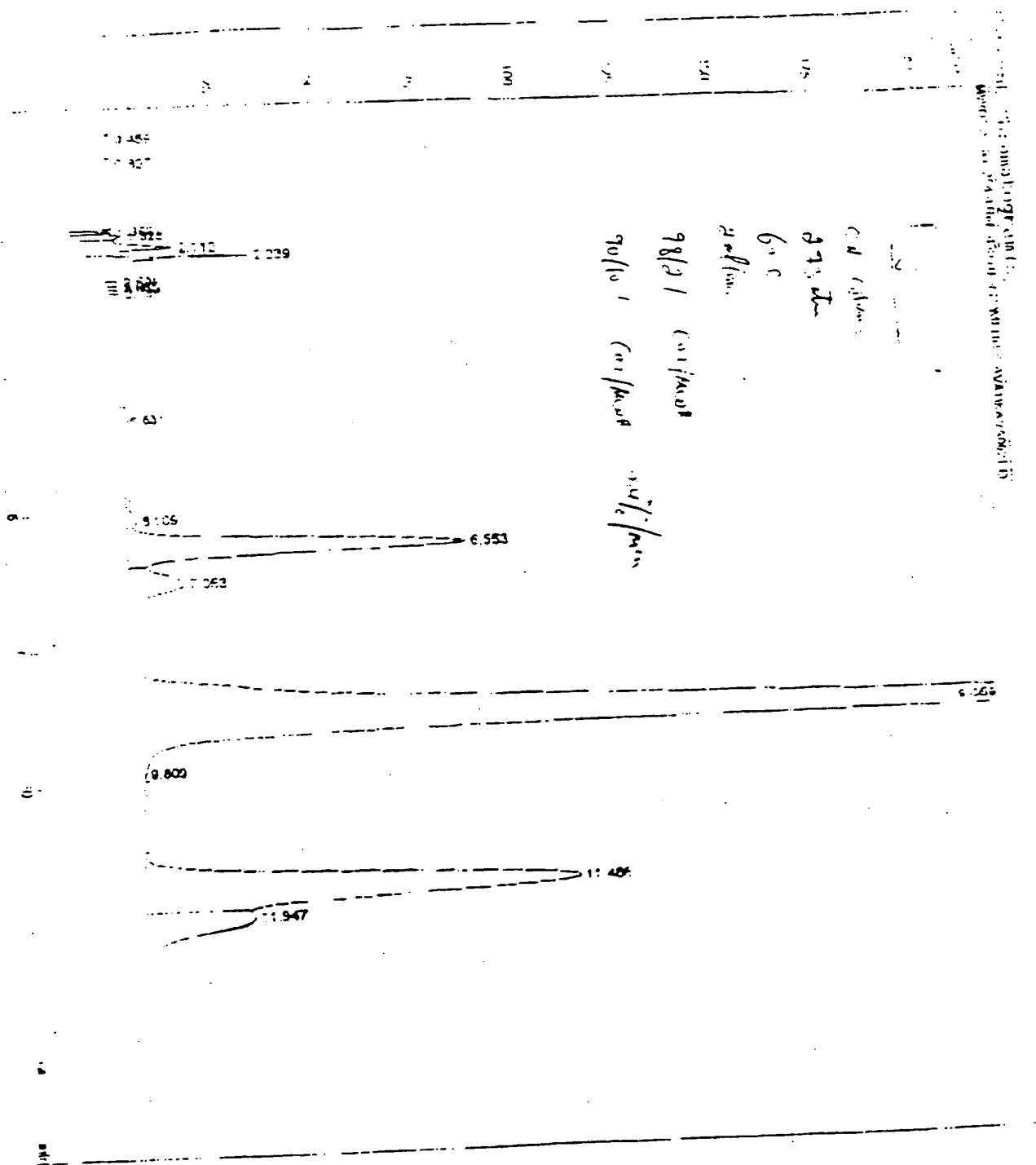


Figure 18

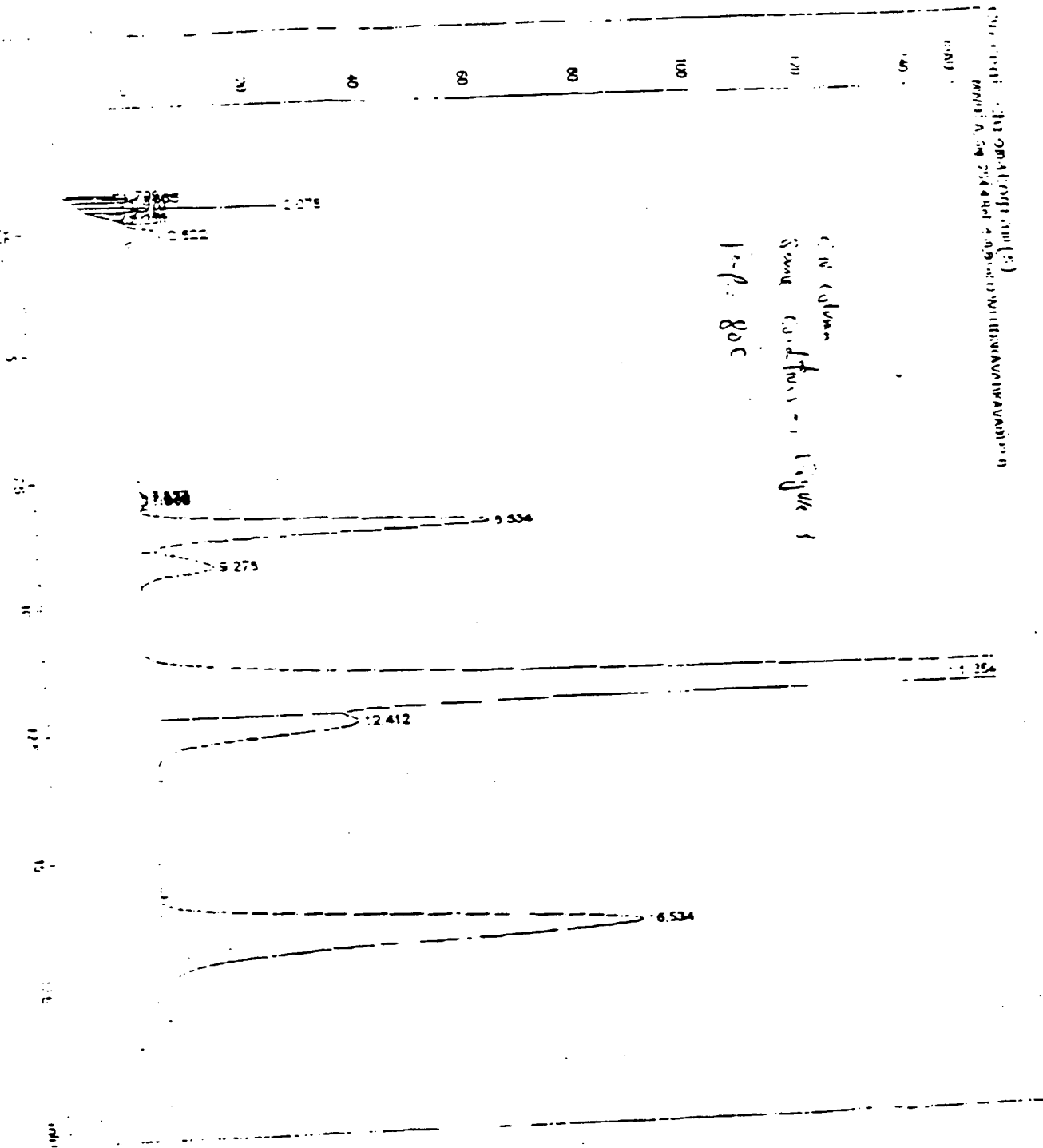
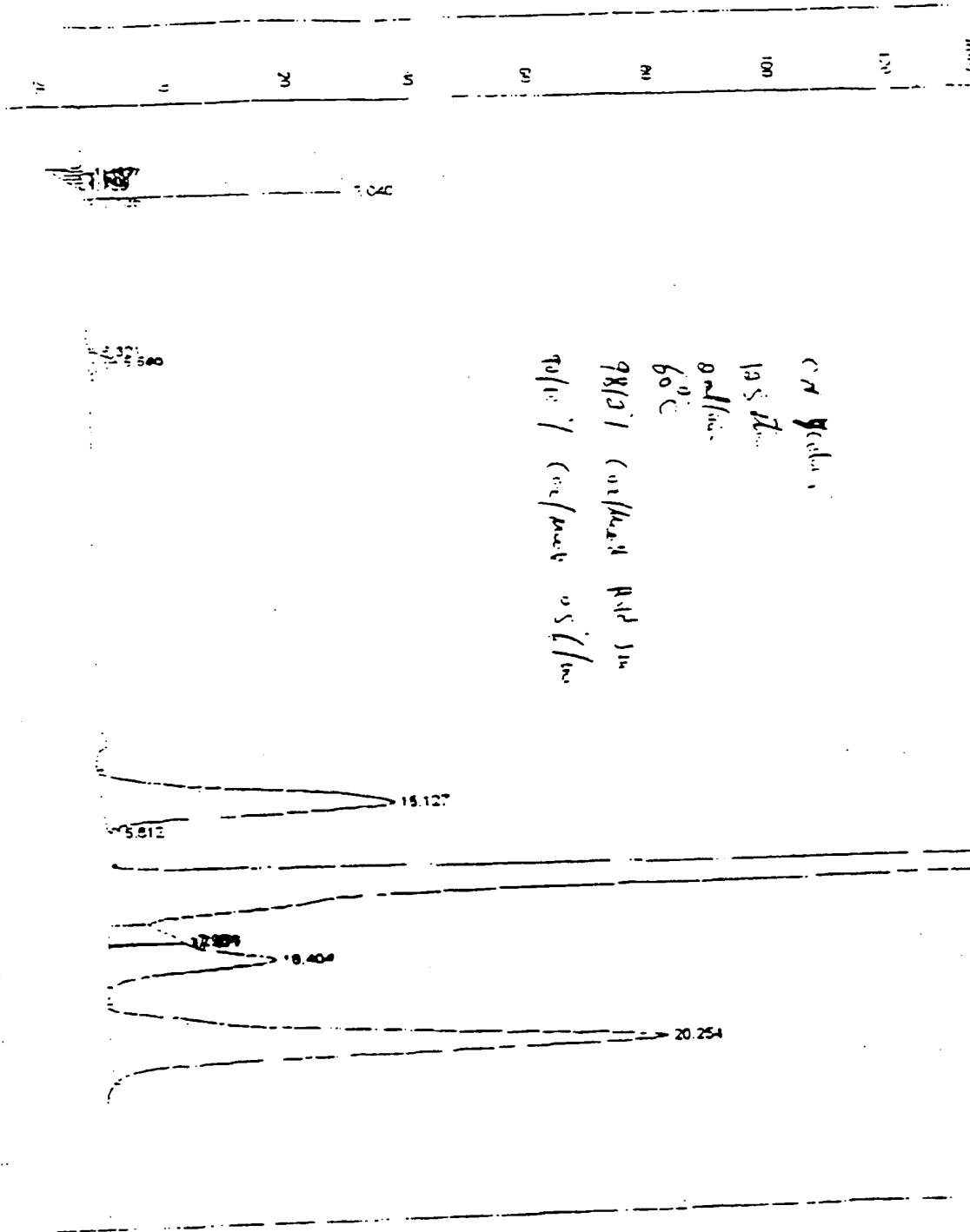


Figure 19

Chemical shift (ppm) vs. intensity



cm⁻¹ 1350
 800
 600
 980/1 (out/line) 1412 1412
 900/1 (out/line) 1412 1412

Figure 20

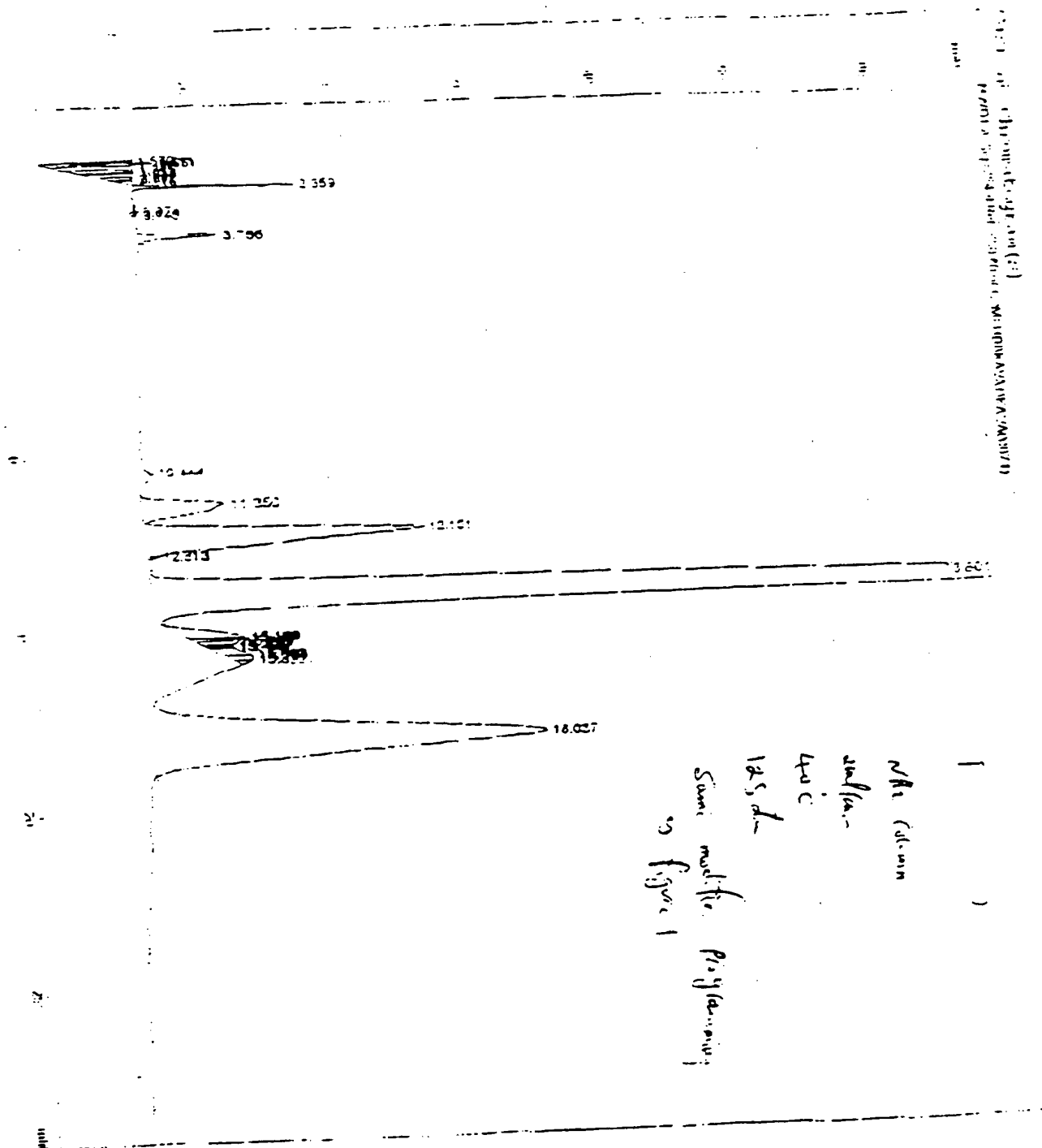


Figure 21

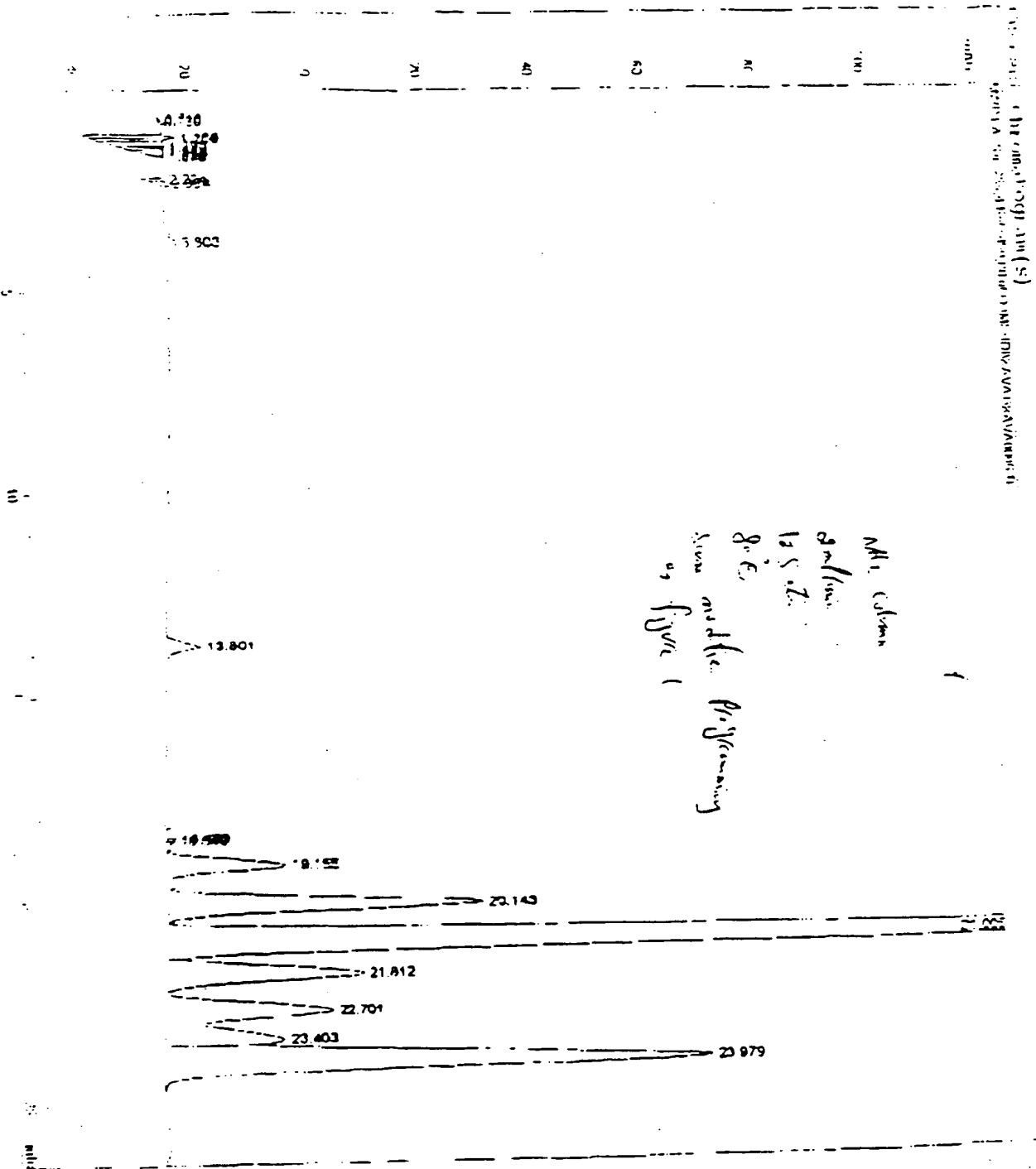


Figure 24

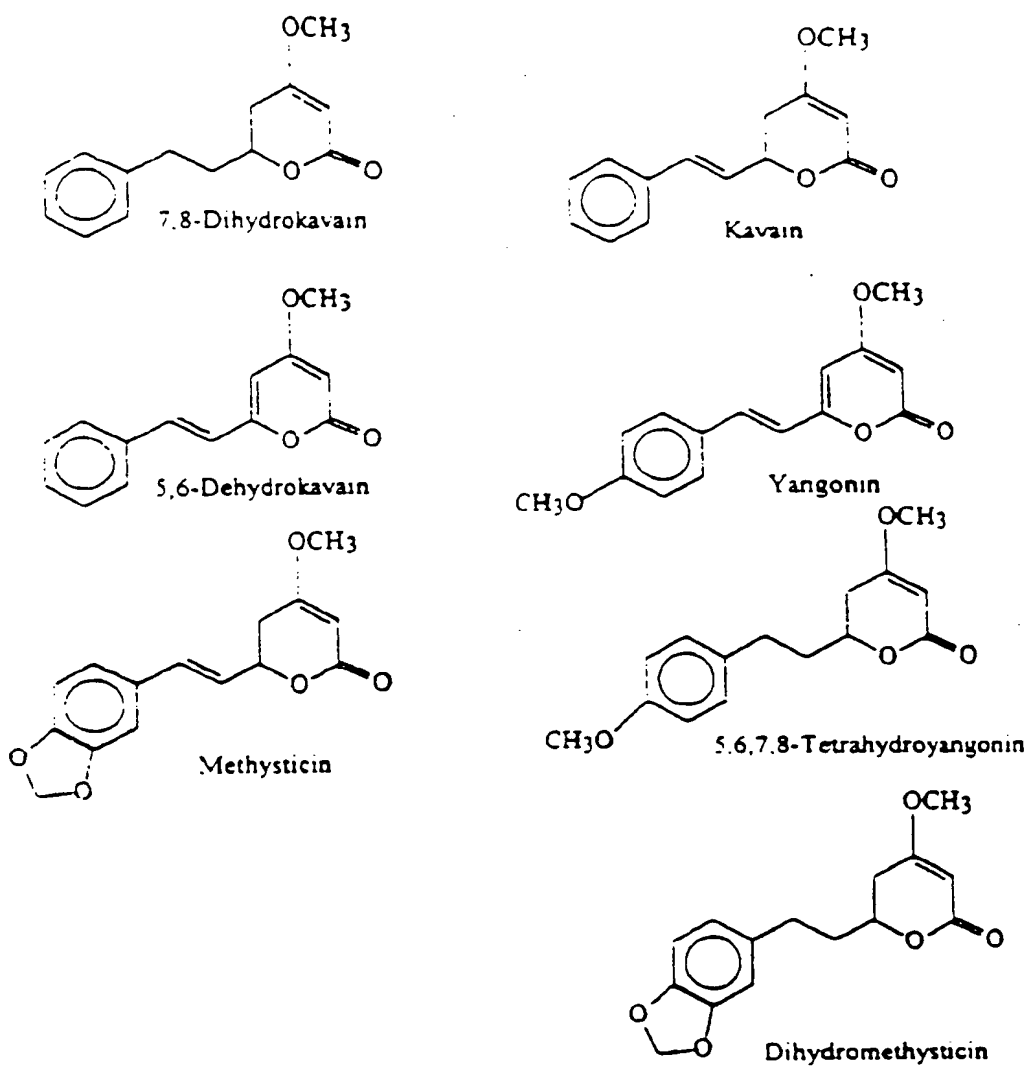


Figure 24

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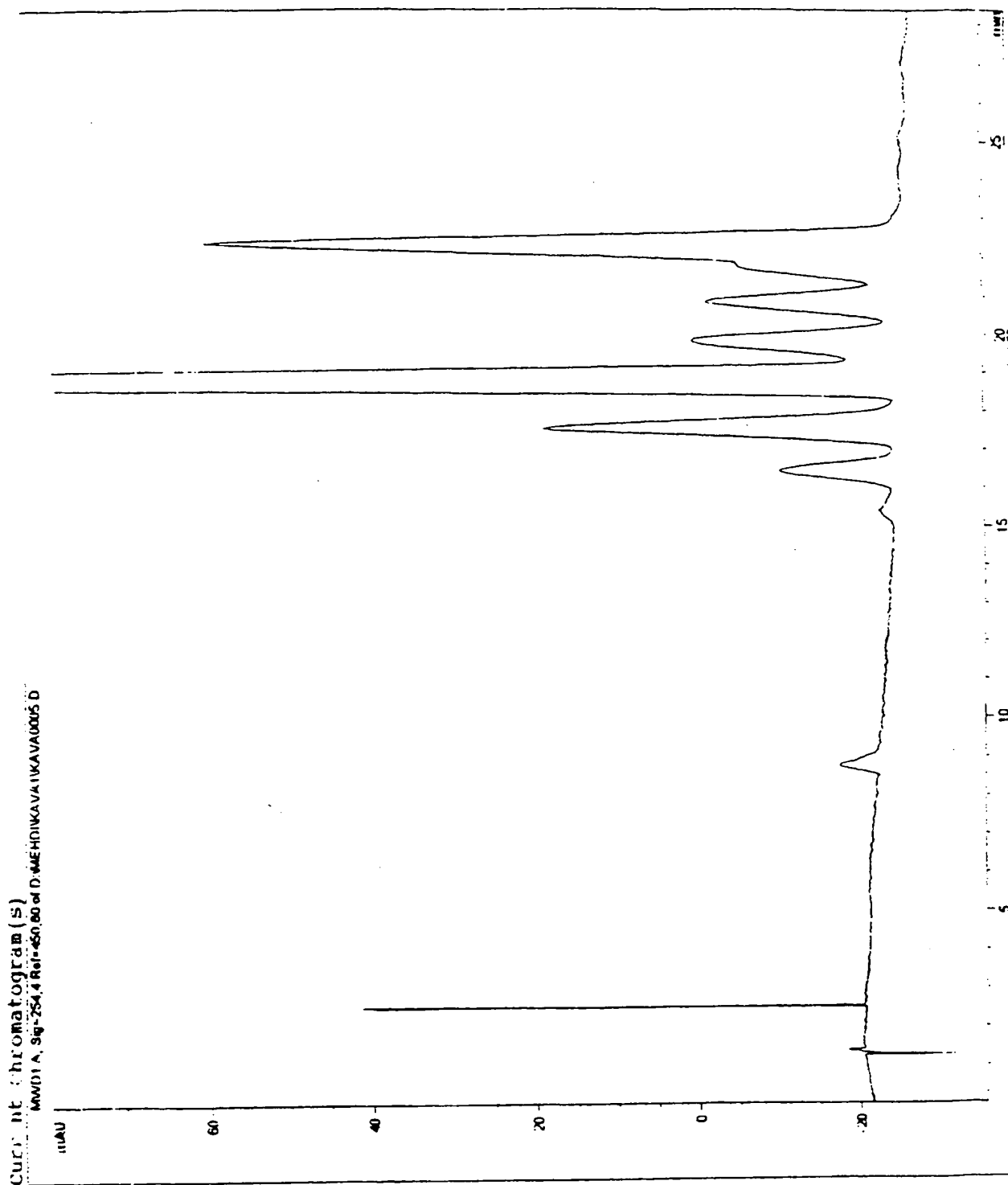


Fig. 25

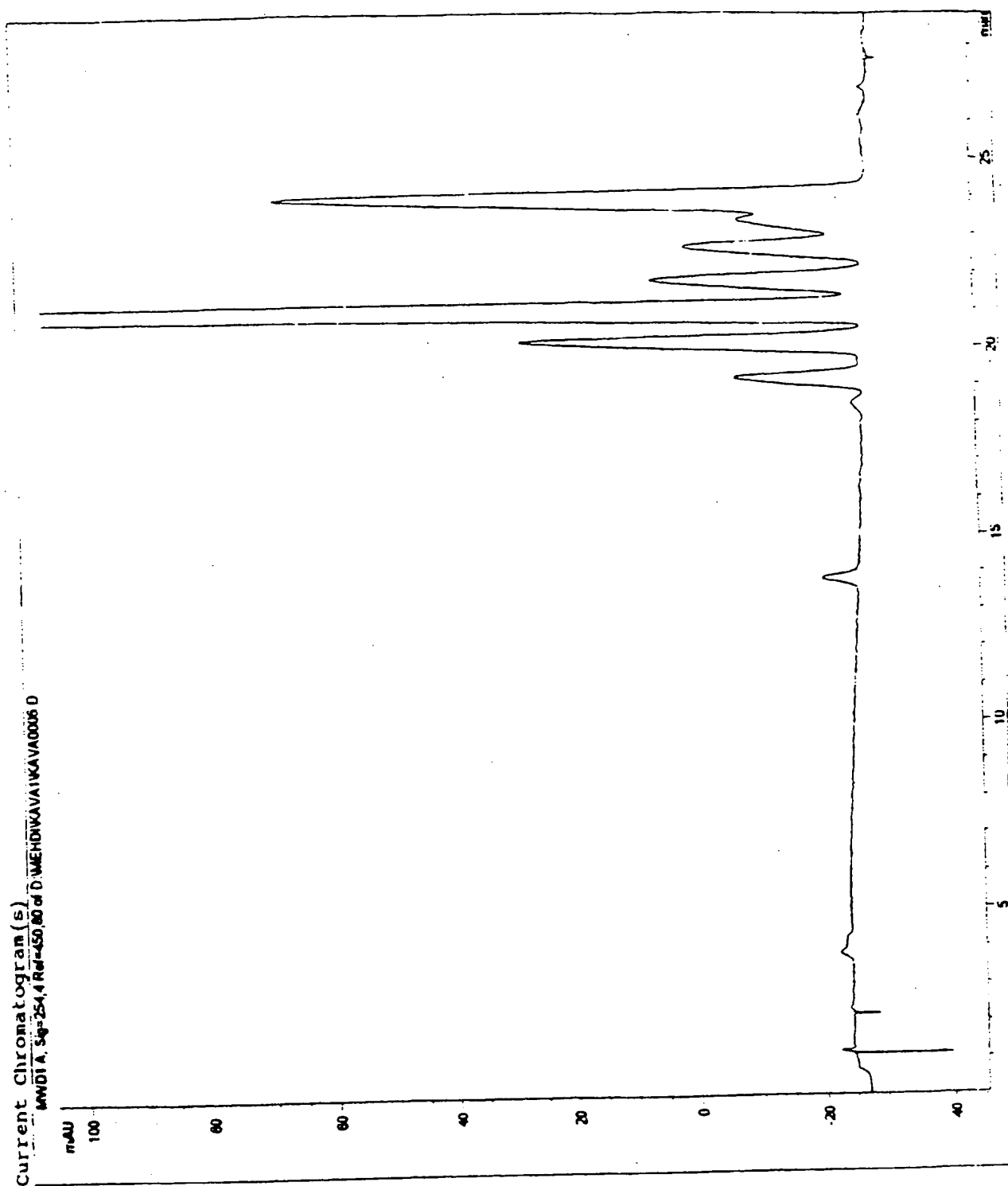


Fig. 26

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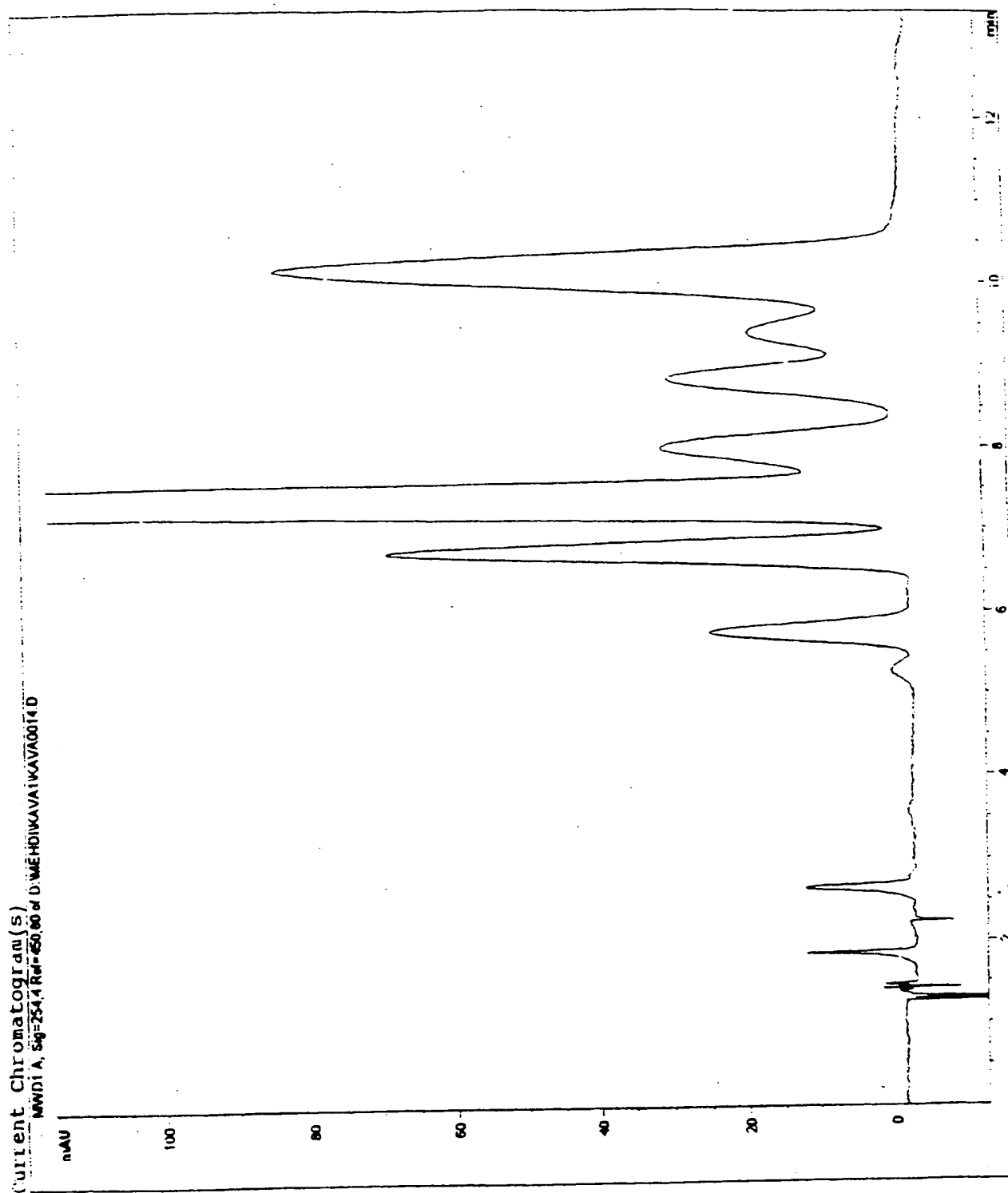


Fig. 27

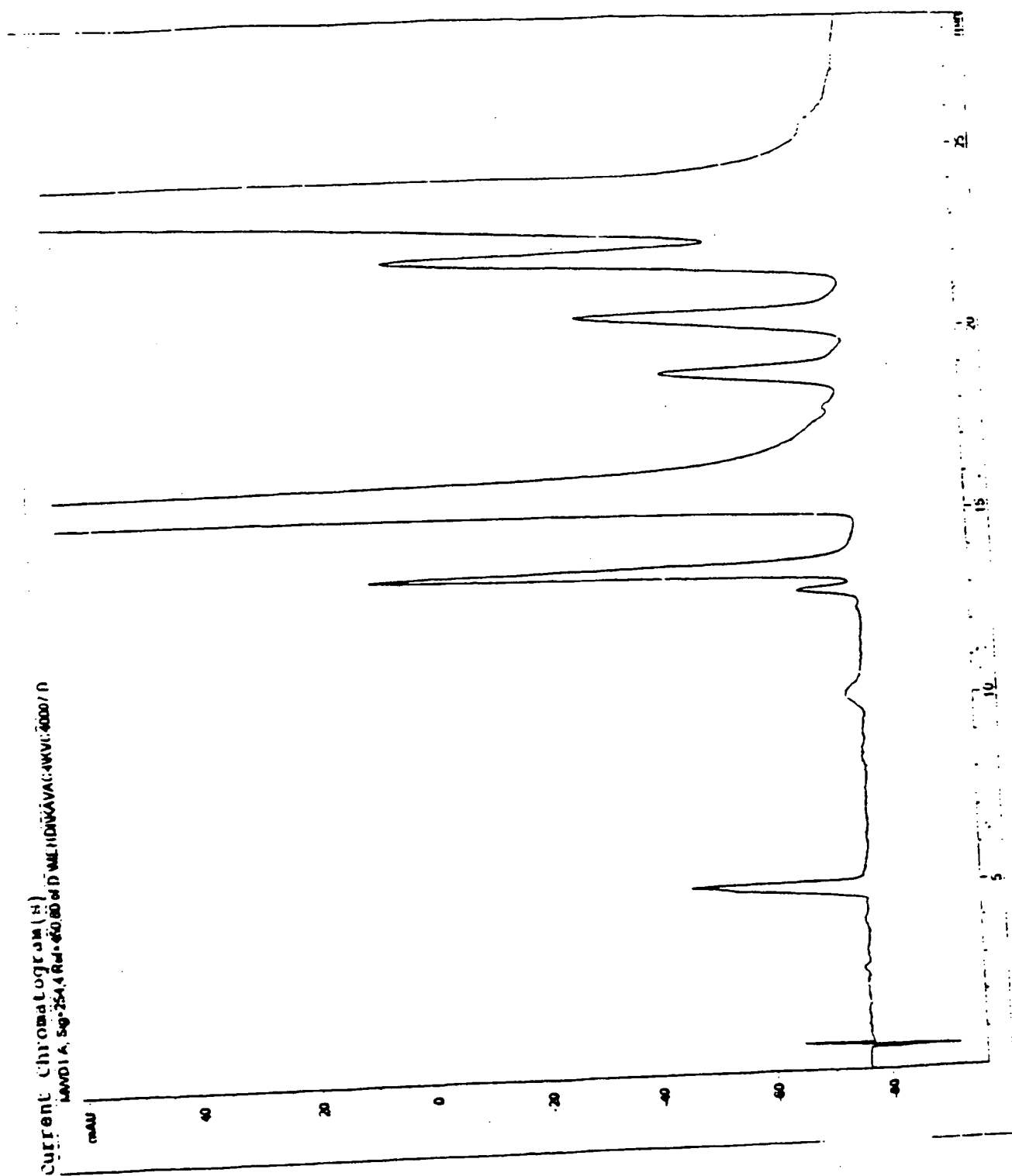


Figure 28

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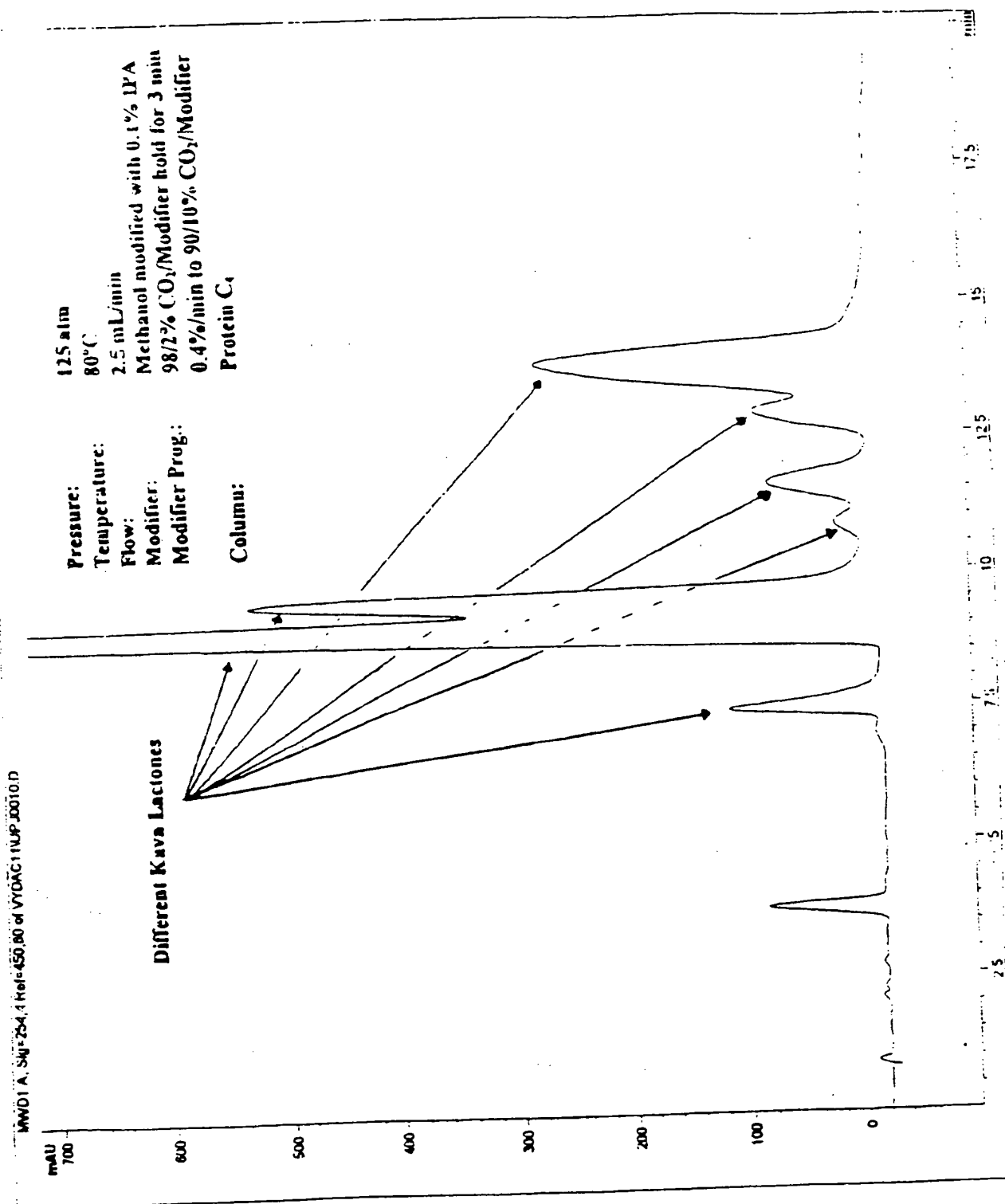


Fig. 29

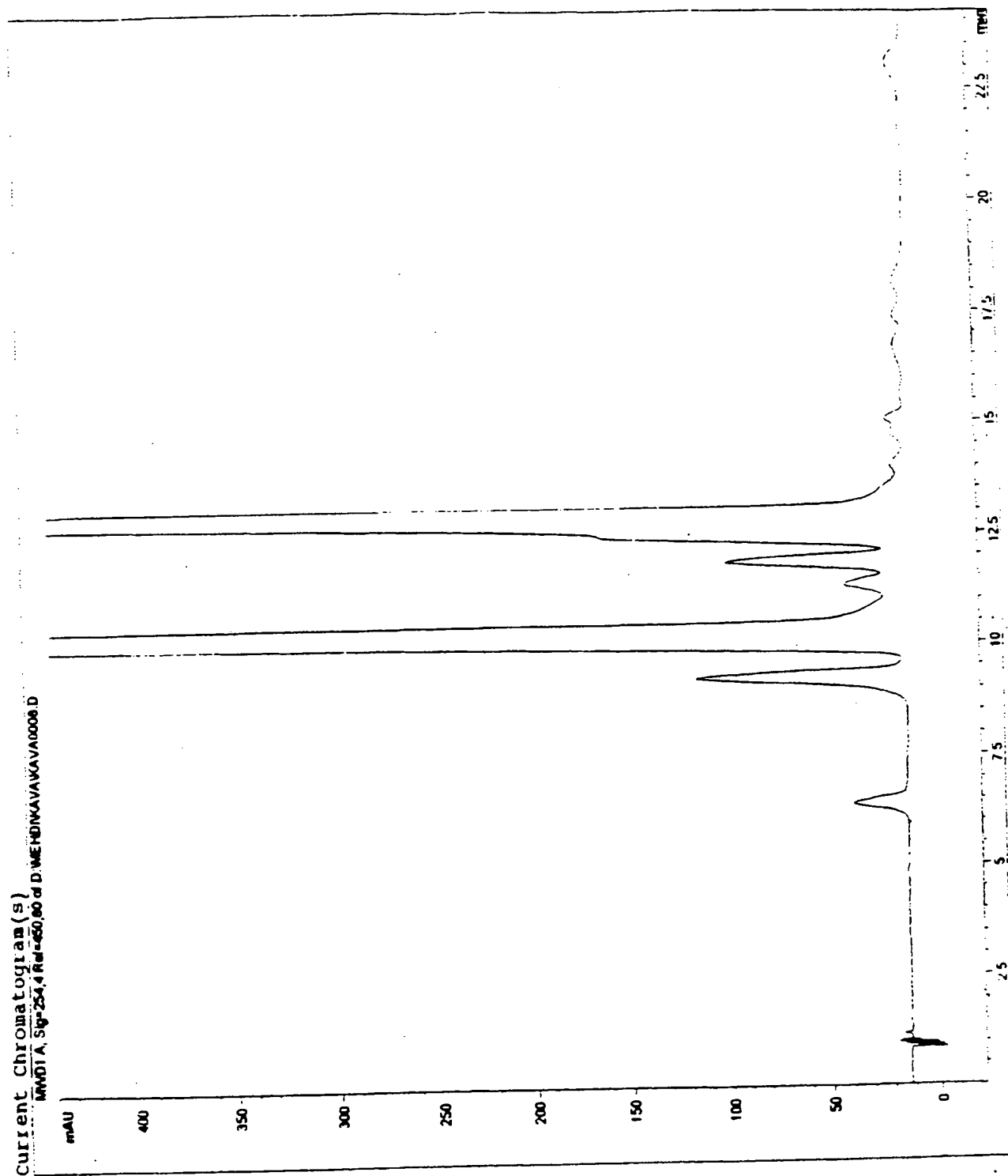


Fig. 30

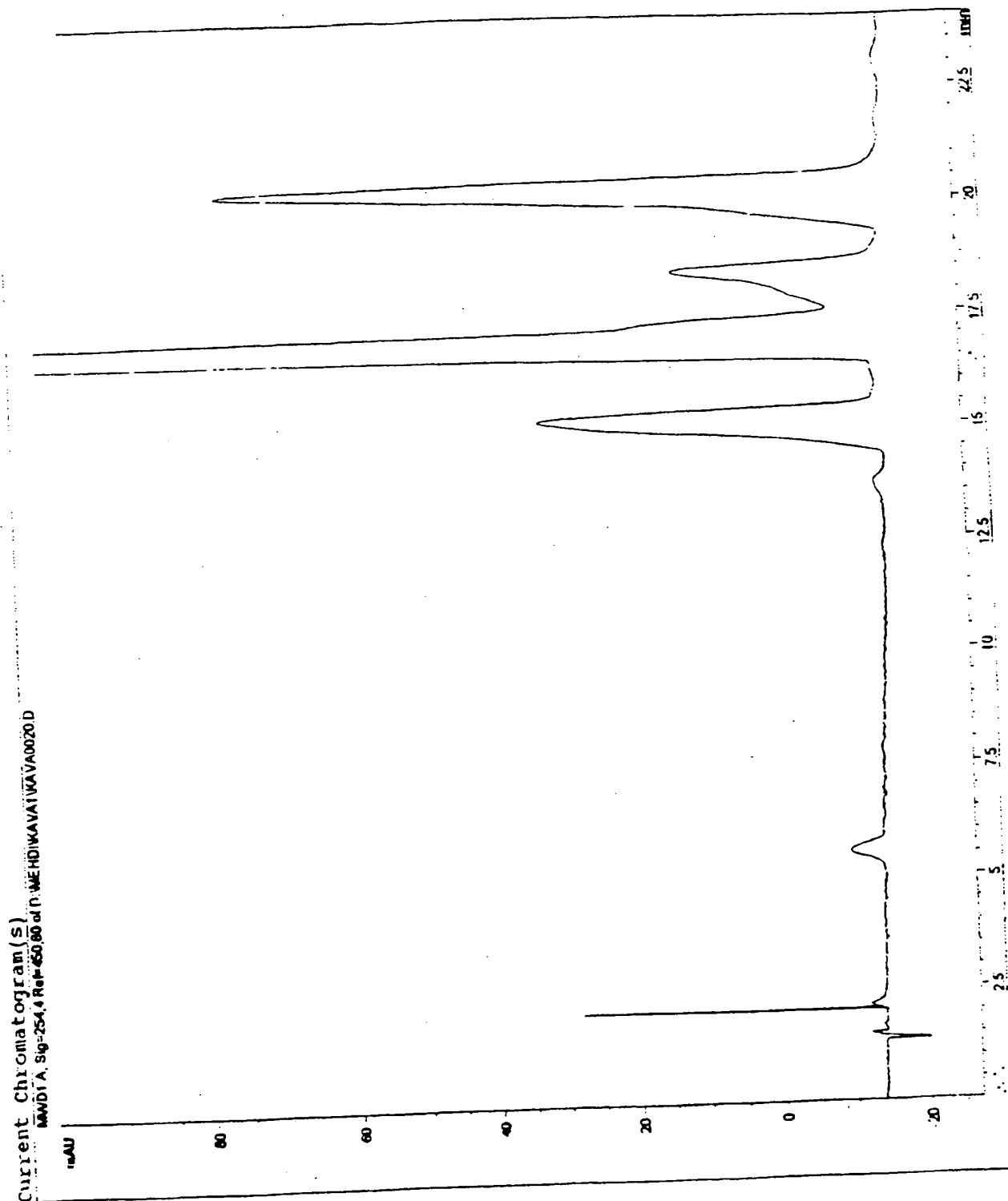


Fig. 31

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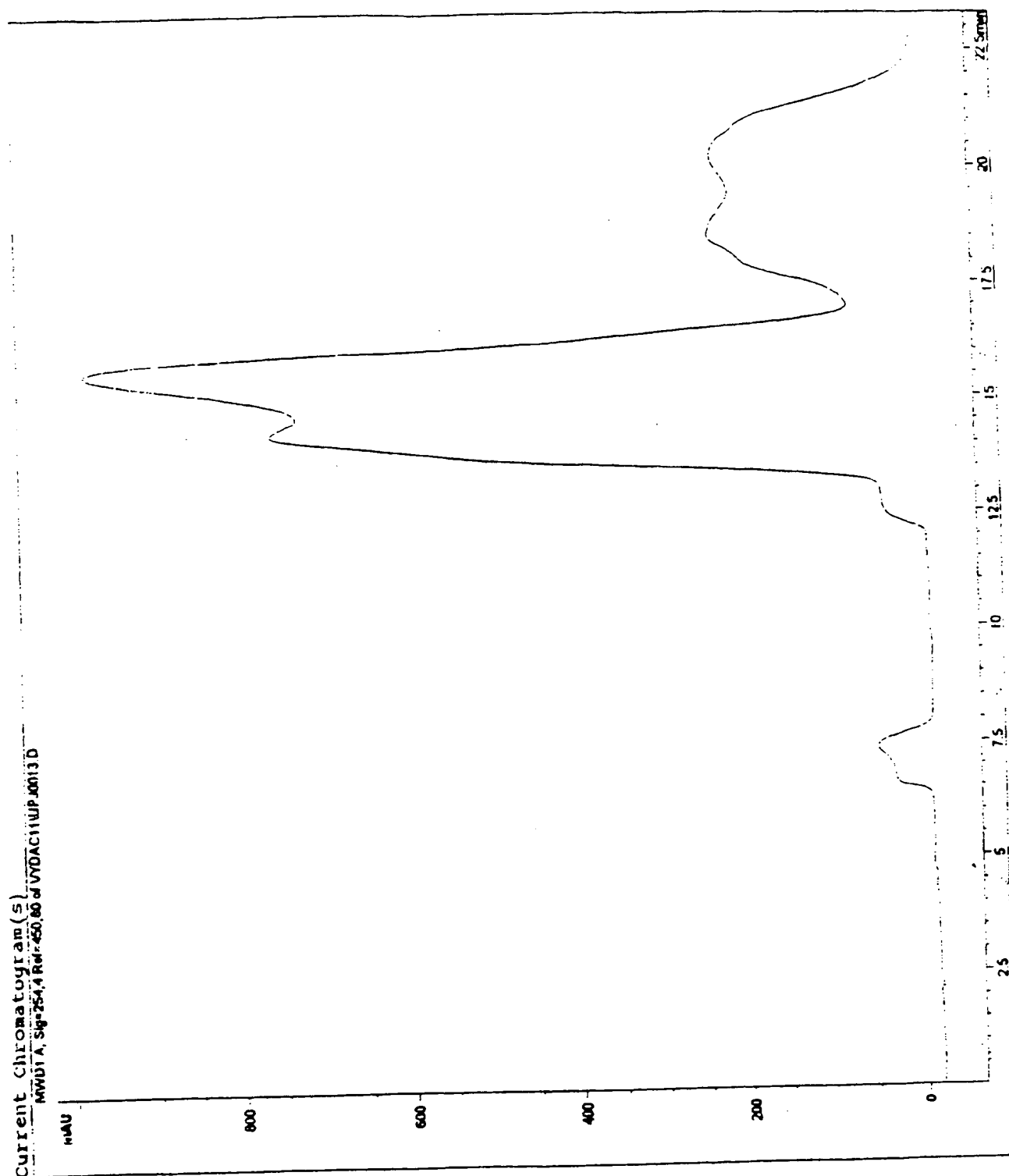


Fig. 32

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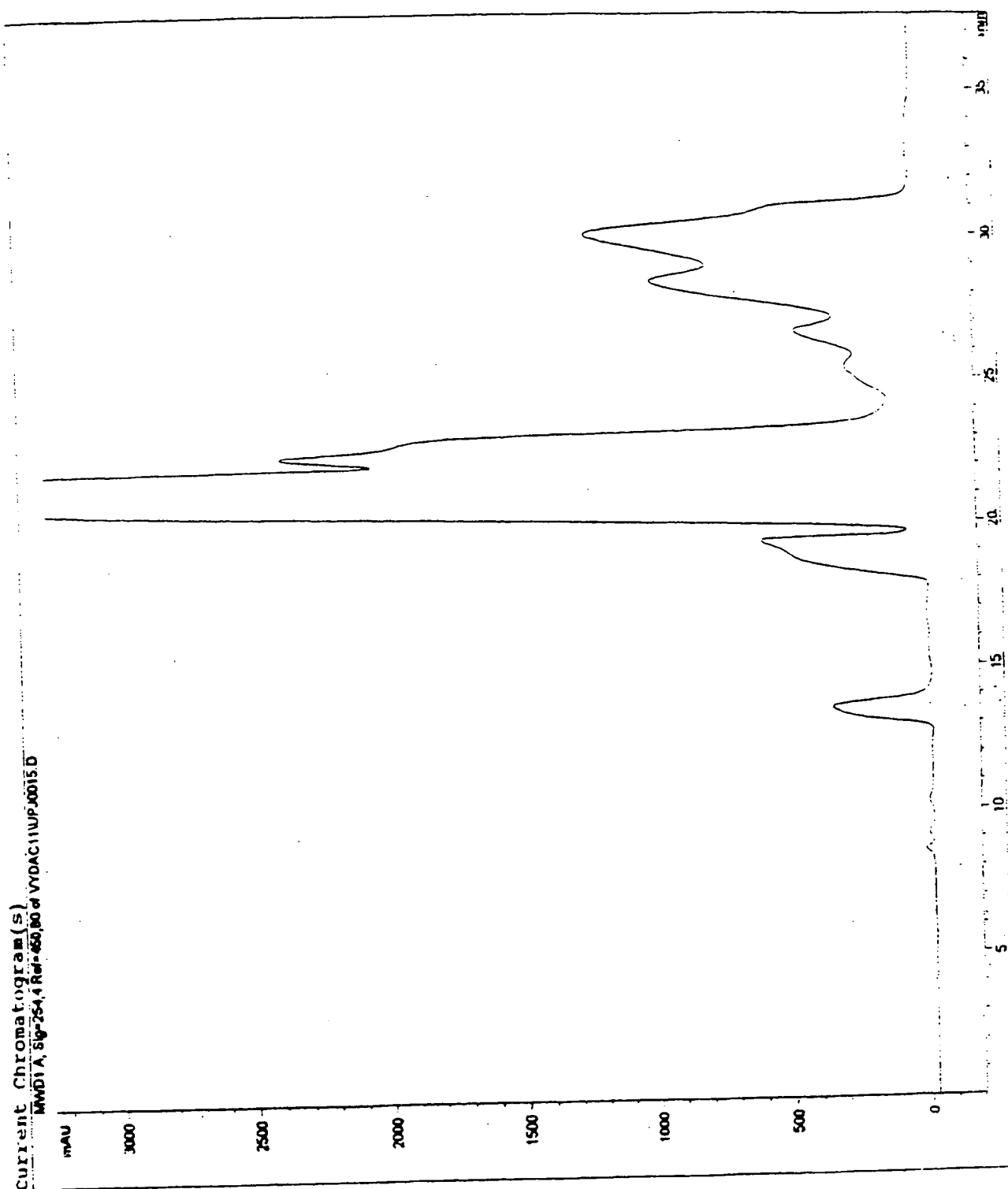


Fig. 33

INTERNATIONAL SEARCH REPORT

Interr val Application No
PCT/US 00/14503

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K35/78 A61P25/00 A61P9/00 A61P3/00 A61P15/00
A61P29/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, FSTA, MEDLINE, PASCAL, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 26794 A (ICI PLC ; POWELL RICHARD LLEWELLYN (GB); NAOKEs TIMOTHY JAMES (GB);) 12 October 1995 (1995-10-12) page 2, line 9 -page 7, line 34 ---	1,3-15, 17-19, 21,23-33
X	GB 2 288 552 A (ICI PLC) 25 October 1995 (1995-10-25) page 2, line 9 -page 7, line 31 -----	1,3-15, 17-19, 21,23-33



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents :

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- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"8" document member of the same patent family

Date of the actual completion of the international search

23 October 2000

Date of mailing of the international search report

30/10/2000

Name and mailing address of the ISA

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Fax: (+31-70) 340-3016

Authorized officer

Rempp, G

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 00/14503

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9526794 A	12-10-1995	AU 678104 B	15-05-1997
		AU 1897095 A	23-10-1995
		BR 9507212 A	09-09-1997
		CA 2185422 A	12-10-1995
		CN 1147208 A	09-04-1997
		EP 0752903 A	15-01-1997
		JP 9510913 T	04-11-1997
		NZ 281989 A	27-05-1998
GB 2288552 A	25-10-1995	NONE	

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